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Begin the article with a short summary.

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Submit an original and three copies of the manuscript, typed double spaced. Each page should begin with a new paragraph. Use accepted spelling, abbreviation, and hyphenation found in *Webster's New International Dictionary*. Footnotes should be typed at the bottom of the page on which they appear.

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Proceedings of the

National Prostatic Cancer Workshop

Bethesda, Maryland

June 28–29, 1976

The National Prostatic Cancer Project is an organ site program of planned and coordinated research supported by the Division of Cancer Research Resources and Centers (DCRRC) of the National Cancer Institute (NCI). This work is supported in part by Public Health Service grant CA-14716.

The objectives of this Workshop are:

a. To exchange information between investigators from different disciplines but with a common denominator of allied interests.

b. To generate ideas for participants and Working Cadre members of the National Prostatic Cancer Project in terms of direction to be taken in current and proposed research areas.

c. To publicize the program, stimulate interest in prostate physiology, and provide a background of information to new investigators in the field.

d. The Workshop will serve to coordinate the scientific activities of all grantees under this program.

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Introductory Remarks

For the past 4 years, a national commitment for a comprehensive, multidisciplinary assault on the grave problem of prostatic cancer has been organized and supported by the National Cancer Institute through an organ site grant-supported program of research in the Division of Cancer Research Resources and Centers. From the outset, efforts have been sustained to assure the highest possible quality of both the scientific as well as the clinical aspects of this endeavor. This workshop represents the second national report to the medical community at large of the significant progress attained towards the management of prostatic cancer in man. This national effort has not been confined to those laboratories or institutions receiving direct grant support from the program. Indeed, as the list of participants and reports demonstrates, continuous exchange of information, development of new projects, and formulation or modification of new treatment trials has been a national as well as an international hallmark of this program. Significant progress in the areas of epidemiology, etiology, diagnosis, and treatment of prostatic cancer are documented in this issue. However, this report is confined to the papers themselves and for the sake of time and space does not include the extensive discussion and exchange of information between the participants.

Gerald P. Murphy

National Prostatic Cancer Project

Although it has been almost 3 years since I was formally associated with the National Prostatic Cancer Project, I have continued to follow its accomplishments. The clinical cooperative chemotherapy trials and the associated drug development and screening programs which this project spawned are offering new alternatives to the standard treatment of prostatic cancer employed in past decades. Preclinical studies supported by the project are painstakingly contributing toward a better understanding of the normal and diseased prostate and promise to offer new clues toward the etiology and early diagnosis of the malignant state.

The increased interest in prostatic research that has emerged in the past few years has in no small measure been engendered by the activities of the National Prostatic Cancer Project. This attests to the success of the organ site approach wherein preclinical and clinical scientists have joined together, with increased understanding and mutual professional respect, in formulating a multidisciplinary attack on this disease. It is this totality of effort combined with the responsibility for identifying priority areas of research that contributes to the uniqueness of these projects.

It is particularly significant that the organ site programs offer the opportunity to successfully pursue targeted research initiated by investigators who design, execute, and interpret new investigative approaches. Despite the growing pains that are inevitable in developing a new approach to the problem of high-incidence cancer, the organ site program approach is proving to be sound.

Thomas J. King

Division of Cancer
Research Resources and Centers
National Cancer Institute

RNA Tumor Virus-Like Activities in Human Prostate: Possible Novel Pharmacologic Approaches [1]

S. K. Arya,* L. Job, J. S. Horoszewicz, R. F. Zeigel, and W. A. Carter [2]

SUMMARY

The cytoplasmic extracts of human prostatic tissues yield two classes of "particles" when centrifuged to equilibrium in a sucrose density gradient, one class banding at a density of 1.15–1.18 g/cm³ ("high density particles") and another at a density of 1.07–1.14 g/cm³ ("low density particles"). Both bands display endogenous DNA polymerase activity which is largely resistant to actinomycin D inhibition. The endogenous DNA products synthesized by high density particles give some indication of high molecular weight RNA:DNA complexes. The tissue extracts from normal, hyperplastic, and neoplastic prostate behave similarly in these assays. In addition, explant cultures of hyperplastic and neoplastic prostate either release, or can be induced to release, particles by treatment with bromodeoxyuridine. These particles band at a density of 1.15–1.18 g/cm³ in a sucrose density gradient and possess RNA and associated DNA polymerase activity which utilizes poly(A):oligo(dT). Our results suggest that human prostatic tissues may contain functions analogous in some ways to those of known RNA tumor viruses of other species.

[Cancer Treat Rep 61:113–117, 1977]

RNA tumor viruses, particularly with C-type morphology, regularly cause neoplasia in lower animals. These findings have now been extended to subhuman primates as well. The association of C-type viruses with some human neoplasms has also been noted, for example, the recent isolation of a C-type virus from cultures of human myelogenous leukemia cells (1). In addition, some evidence for the association of subviral components, related to known RNA tumor viruses, with several human malignancies has been obtained in recent years (1,2).

These observations argue for the possible association of RNA tumor viruses with human cancers including prostatic carcinoma. We are investigating such an association utilizing a battery of complementary approaches (3). We have recently reported the detection of particle-associated virus-like activities in several human prostatic tissue specimens (4). We have also found that the explant cultures of human prostatic tissues can be induced to produce "particles" possessing virus-like activities. We briefly review these results here. Our studies, in addition to providing possible clues to etiology, may also lead to the development of some new prognostic and therapeutic modalities. For example, purified human interferon may prove to be a selective antiproliferative agent for human prostatic carcinoma cells. We are now determining if purified human interferon shows selective cytotoxicity for cells and explant cultures derived from neoplastic prostate.

MATERIALS AND METHODS

Procurement and processing of tissue specimens.— Prostatic tissue specimens were obtained from Buffalo area hospitals and the Prostatic Tissue Bank, University of Miami. The diagnosis was established by resident pathologists in the respective institutions. The procedures for preparing cytoplasmic extracts and subsequent analysis of the extracts for particle-associated endogenous DNA polymerase activity have been described previously (3,4).

[1] Supported in part by Public Health Service grant CA-15502 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Department of Medical Viral Oncology, Roswell Park Memorial Institute, and Graduate Faculty in Microbiology, State University of New York, Roswell Park Division, Buffalo.

*Reprint requests to: Dr. S. K. Arya, Department of Medical Viral Oncology, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

TABLE 1.—Equilibrium banding of prostatic cytoplasmic extracts and sedimentation of endogenous DNA products*

Type	Source	Particles with DNA polymerase activity at—		DNA products synthesized by particles at 1.15–1.18 g/cm³	
		1.15–1.18 g/cm³	1.07–1.14 g/cm³	RNA:DNA	DNA
Murine leukemia virus	Cell culture	+	−	+	+
Moloney leukemia virus-infected murine cells	Cell culture	+	±	+	+
Normal prostate	Autopsy	+	+	ND	ND
Normal prostate	Autopsy	+	+	±	+
Hyperplastic prostate	Autopsy	+	+	±	+
Hyperplastic prostate	Surgery	+	+	−	+
Neoplastic prostate	Autopsy	+	+	±	+
Neoplastic prostate	Autopsy	+	+	±	+

* + = activity or entity was observed; − = activity or entity was not observed; ± = activity or entity was indicated but its level was too low to reach a definitive conclusion. ND = not determined.

Explant cultures and bromodeoxyuridine (BUdR) treatment.—Thirty to 35-day-old explant cultures were treated for 3 days with 50 μg/ml of BUdR. The cultures were then incubated with fresh medium containing 10 microcuries/ml of [³H]uridine. Twenty-four hours later, the culture medium was harvested and processed for detecting virus-like activities as described before (3,4).

RESULTS AND DISCUSSION

RNA Tumor Virus-Like Activities in Prostatic Tissue Extracts

RNA tumor virus particles contain RNA and associated DNA polymerase. These particles, when incubated in vitro with DNA precursors in the presence of a nonionic detergent, synthesize DNA directed by the resident template RNA (the endogenous DNA polymerase reaction). When such an endogenous reaction was carried out with cytoplasmic extracts of human prostatic tissues, a significant endogenous DNA polymerase activity was elicited. This activity was largely resistant to actinomycin D inhibition, suggesting that it was RNA-directed. To characterize this activity further and to determine if the activity was associated with particles of density similar to that of RNA tumor viruses, aliquots of prostatic cytoplasmic extracts were banded in isopycnic sucrose gradients. As shown in figure 1, the DNA polymerase activity separates into two broad bands, one corresponding to a density of 1.15–1.18 g/cm³ ("high density particles") and the other to a lower density of 1.07–1.14 g/cm³ ("low density particles"). The two broad bands were observed consistently in all similarly processed tissues from the normal, hyperplastic, or neoplastic prostate (table 1). The DNA synthesized by high density

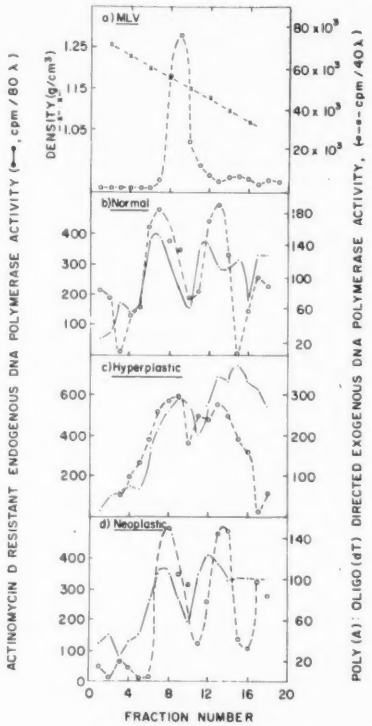
a) MLV
b) Normal
c) Hyperplastic
d) Neoplastic
DENSITY (g/cm³ x 10³)
ACTINOMYCIN D RESISTANT ENDOGENOUS DNA POLYMERASE ACTIVITY (●—●, cpm / 80 λ)
POLY (A) · OLIGO (dT) DIRECTED EXOGENOUS DNA POLYMERASE ACTIVITY, (▲--▲, cpm / 40 λ)
FRACTION NUMBER

FIGURE 1.—Banding of cytoplasmic extracts of prostatic tissue specimens in isopycnic sucrose gradients. An aliquot of cytoplasmic extract was layered on a 15%–60% sucrose gradient and centrifuged for 18 hrs at 26,000 rpm in a Spinco SW 50.1 rotor. Aliquots of fractions were used for determining the endogenous and exogenous DNA polymerase activity, the latter directed by poly(A):oligo(dT). Polymerase activity of (*a*) purified Moloney murine leukemia virus (MLV); (*b*), (*c*), and (*d*) cytoplasmic extracts of prostatic tissue specimens.

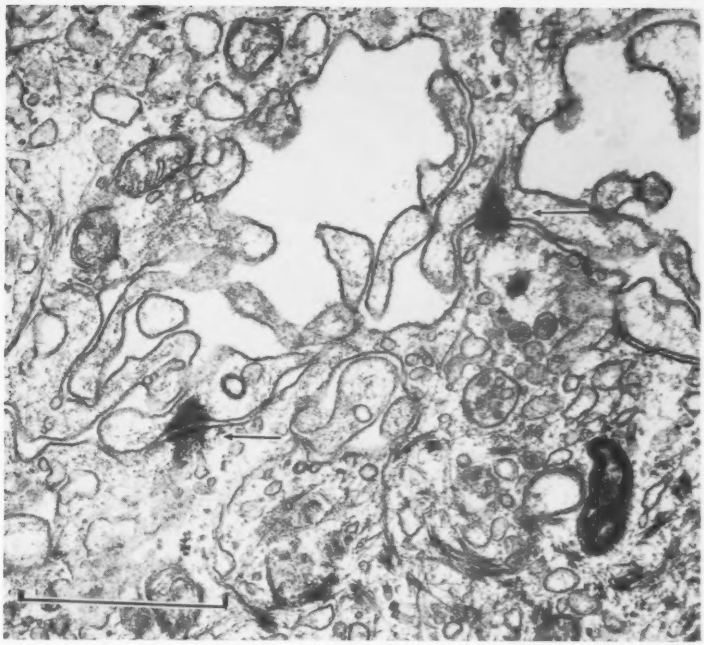

FIGURE 2.—Portions of 2 adjacent cells at 27 days in culture derived from a human hyperplastic prostate gland. 2 desmosomes (arrows) are observed and cytoplasmic tonofibrillae appear, characteristically, to insert in the submembrane region of the desmosomes suggestive of the epithelioid nature of these cells. Bar = 1 μm.

particles was further analyzed to determine if it contained high molecular weight RNA:DNA complexes. Only limited evidence for such complexes was obtained (*see* table 1 and ref 4). This may be due to an enrichment of the prostatic cytoplasmic extracts with nucleases, as apparently occurs in the rat ventral prostate (G. Williams-Ashman, personal communication, 1975). Thus, these results suggest that prostatic tissue extracts, independent of their normal or malignant character, may contain entities at least similar in two respects to those of C-type RNA viruses, namely, they display an actinomycin D-resistant endogenous DNA polymerase activity and a buoyant density behavior usually associated with RNA tumor viruses.

However, the possibility that the observed activity may be due to chromatin fragments or other artifactual nucleic acid:polymerase complexes has not yet been ruled out. Thus, it is not yet clear if the activities we have noted are in fact viruses or subviral components and if they also reside in normal human prostate. It is to be noted that Gallo and co-workers (1) also recently reported similar observations of high and low density particle-associated activities in normal and leukemic human cells; in fact, their density gradient profiles are quite similar to those we observed for prostatic tissues. They were able to show that the high density particles activity of leukemic cells was RNA-directed and synthesized DNA which contained sequences hybridizable to simian sarcoma virus RNA. In contrast, the high density particles activity of normal cells was DNA-directed and synthesized DNA lacking viral sarcoma-specific sequences. Although we have not yet tested this, it is conceivable that high density particles we obtained from neoplastic and normal prostatic tissues synthesize DNA products with similar nucleotide sequence differences. This possibility is now being examined.

Induction of RNA Tumor Virus-Like Activities in Human Prostatic Explant Cultures

We have recently found that explant cultures (fig 2) of prostatic tissues can be induced to produce virus-like activities by treatment with halogenated

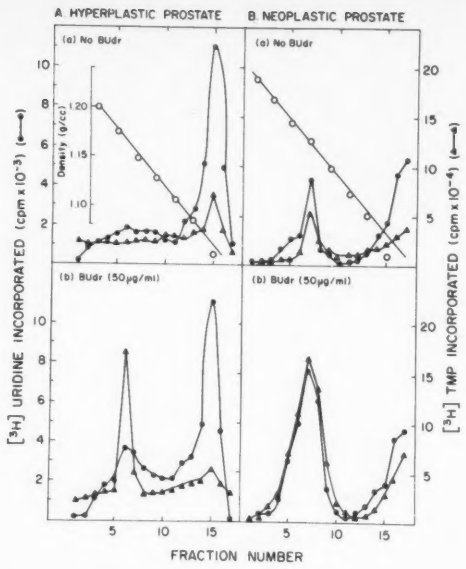

FIGURE 3.—Induction of RNA tumor virus-like particles in human prostatic explant cultures by BUdR. Explant cultures were treated with BUdR (50 μg/ml) for 3 days and refed with fresh medium containing [^{3}H]uridine (10 microcuries/ml). 24 hrs later the culture medium was pelleted through a column (20% glycerol) and suspended pellet was centrifuged to equilibrium in a 15%–60% sucrose gradient. An aliquot of fractions was TCA-precipitated to measure the incorporation of [^{3}H]uridine into RNA and another aliquot was used to estimate DNA polymerase activity catalyzed by poly(A):oligo(dT).

pyrimidines. When 30–35-day-old explant cultures of hyperplastic and neoplastic prostate were treated with BUdR, they produced particles which banded at a density of 1.15–1.18 g/cm^3 in equilibrium sucrose gradients. These particles contained RNA as judged by [^{3}H]uridine incorporated into acid-precipitable material and associated DNA polymerase which utilized poly(A):oligo(dT) (fig 3, table 2). Some of the hyperplastic explant cultures produced particles even prior to BUdR treatment, while in others induction was observed only after BUdR treatment (table 2).

Similar particle-associated activities were observed in explant cultures of neoplastic prostatic tissue specimens which were enhanced by BUdR treatment (fig 3 and table 2). We have not yet determined if similar activities can be induced from explant cultures of normal prostatic tissue specimens. The induction of particle-associated activity from explant cultures is in accord with our observation of similar activities in prostatic tissue extracts. While these activities clearly need to be further characterized, our results support the notion that RNA tumor viruses may be associated with human prostatic tissues.

We are now investigating further the induction of virus-like activities in explant cultures by using purified human interferon as a probe. Interferon has been shown to modulate the expression of virus information (5). It is of interest to determine if human interferon, which we have now purified (6), can suppress the expression of virus information in human prostatic cells. In addition, interferon possesses antiproliferative properties (7). Our preliminary results indicate that the addition of purified human interferon to prostatic epithelial explant and fibroblastic cell cultures results in a dose-dependent reduction of labeled thymidine incorporation when measured by radioautography. This reduction appears to be more marked for epithelial explant than for fibroblastic cell cultures. Studies are now in progress to determine if human interferon displays

TABLE 2.—Productive induction of RNA tumor virus-like particles in human prostatic explant cultures by BUdR

Type	Age of primary explant culture (days)	BUdR (50 μg/ml)	Equilibrium banding in sucrose gradients: [³H]uridine incorporated at 1.15–1.18 g/cm³ (normalized cpm*)	Equilibrium banding in sucrose gradients: Reverse transcriptase activity at 1.15–1.18 g/cm³ † (normalized cpm*)
Hyperplastic	20	–	600	NT‡
		+	20,000	NT
Hyperplastic	30	–	20	NT
		+	2500	NT
Hyperplastic	30	–	0	0
		+	0	0
Hyperplastic	35	–	1900	6600
		+	2500	36,800
Hyperplastic	35	–	6100	83,000
		+	10,900	361,000
Adenocarcinoma	35	–	8000	110,000
		+	21,000	472,000
Adenocarcinoma	35	–	11,000	3600
		+	8500	3500

*Radioactivity incorporated has been normalized with respect to the [³H]uridine incorporated into total culture RNA. Activity is expressed as cpm per million cpm of [³H]uridine incorporated by the explant culture.

†[³H]thymidine monophosphate incorporated in an exogenous reaction directed by poly(A):oligo(dT).

‡NT = not tested.

differential antiproliferative effects for epithelial cultures derived from neoplastic as compared with normal human prostate.

In summary, we have presented the results of our work toward the assessment of oncornavirus participation in prostatic neoplasia as well as a blueprint for exploiting the antiviral and antiproliferative potential of human interferon. The results of these studies may lead to the development of new prognostic and therapeutic modalities applicable to human prostatic cancer.

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Search for Oncogenic Viruses in Human Prostate Cancer [1]

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SUMMARY

Electron microscopic, immunologic, and biochemical methods have been used in an attempt to detect and characterize oncornaviruses in human prostatic carcinoma (PCa) and benign prostatic hyperplasia (BPH), and in prostates of mice of high and low mammary cancer or leukemia strains. Ultrastructural examination of 37 PCa and nine BPH specimens has revealed the presence of particles resembling type C virus in five cases of PCa and one of BPH, and also two different types of intracisternal virus-like particles in seven other cases of PCa. Type B virus particles have been observed in prostate of old mice of high mammary cancer strains, while type C virus particles have been found in the prostates of most mice of all the ten strains examined. Immunofluorescence tests with sera from patients with PCa and BPH and with cells derived in vitro from PCa have shown that sera of patients with PCa contained antibodies directed mainly against Forssman-like and tumor-related antigens. In immunofluorescence tests of antisera to major proteins of oncornaviruses with cells of PCa and BPH tissues grown in vitro, positive reactions have been obtained with antisera to p30 protein of murine, feline, and simian type C viruses. Fixed immunofluorescence (FIF) tests of sera of PCa (38%) and BPH (25%) and of some normal donors (27%) gave positive cytoplasmic reaction with mouse prostate cells infected with Soehner-Dmochowski murine sarcoma virus (SD-MSV). Immunoferritin tests of 11 sera positive by FIF gave ferritin labeling of type C virus particles in the SD-MSV-infected mouse prostate cells. Biochemical studies have shown that cells of a long-term tissue culture from PCa and human fetal prostate released particulate components containing 70S RNA. Molecular hybridization experiments with radioactive DNA probes synthesized by MSV-M, MSV-Ki, SSV type C viruses, and cellular RNA extracted from five PCa, three BPH, and two normal prostates have given low levels of hybridization with MSV-M and MSV-Ki murine viruses. Results of these studies, although preliminary, indicate that a combined ultrastructural, immunologic, and biochemical approach to the problem of viruses and human prostate cancer may lead to positive findings of significance.

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In a search for the possible involvement of oncogenic (either RNA or DNA) viruses in human prostatic carcinoma, a multifaceted approach has been employed comprising ultrastructural, biologic or tissue culture, immunologic, and biochemical methods. This approach has helped to establish the causative involvement of both RNA and DNA viruses in neoplasms of animals extending from molluscs to subhuman primates (1). This multifaceted approach has been made possible through close collaboration of scientists representing various basic disciplines and clinicians, especially surgeons. These studies have been undertaken with the hope that an understanding of at least some aspects of the origin of human prostatic neoplasia may help to serve the management of this type of neoplasia.

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MORPHOLOGIC STUDIES

Human Prostatic Tissues

Forty-nine specimens of human prostatic tissue, which consisted of 37 cases of carcinoma (PCa), nine cases of benign hyperplasia (BPH), three cases of bladder tumor infiltrating prostatic tissue (BT), and histologically normal parts of the prostate from eight cases of PCa, were examined by electron microscopy using methods previously described (2). Thin sections of selected blocks were checked by Paragon stain for the presence of epithelial elements prior to electron microscopy.

Three morphologically different types of virus-like particles have been observed in the epithelial cells of human prostate neoplasia. They were always observed in the epithelial cells.

The first type were large intracisternal virus-like particles (150–200 nm), single or in a group, and budding (fig 1). They have been observed in seven of 37 cases of PCa, which were composed of four cases of poorly differentiated adenocarcinoma and three cases of undifferentiated carcinoma. They have not been observed in nine cases of BPH, three cases of BT, and eight cases of histologically normal parts of the human prostate.

The second type resembled type C virus particles. They have been observed in five other cases of the 37 cases of PCa and in one of the nine cases of BPH. These particles (figs 2a and 2b), 90–130 nm in diameter, have been observed only in the cytoplasm of epithelial cells of PCa but not in the stromal cells. Budding particles resembling budding type C virus have been seen in one case of PCa and one case of BPH (fig 2c).

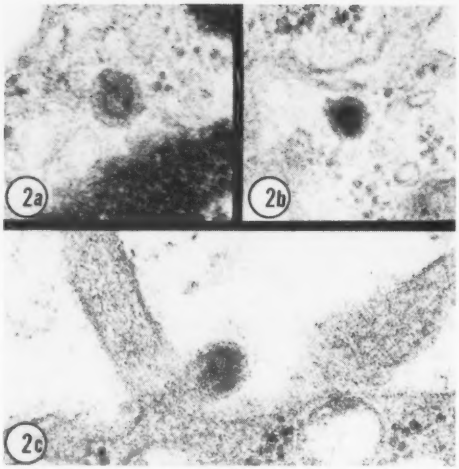

FIGURE 2(a-c).—Budding and particles resembling type C virus in epithelial cells from 3 different cases of human PCa× 60,000).

The third type observed consisted of small particles and budding (fig 3), 80–100 nm in diameter. They have been found only in the rough endoplasmic reticulum of the epithelial cells in one case of PCa together with the first type of virus-like particles. The appearance and pattern of these particles resembled that of hamster type H virus parti-

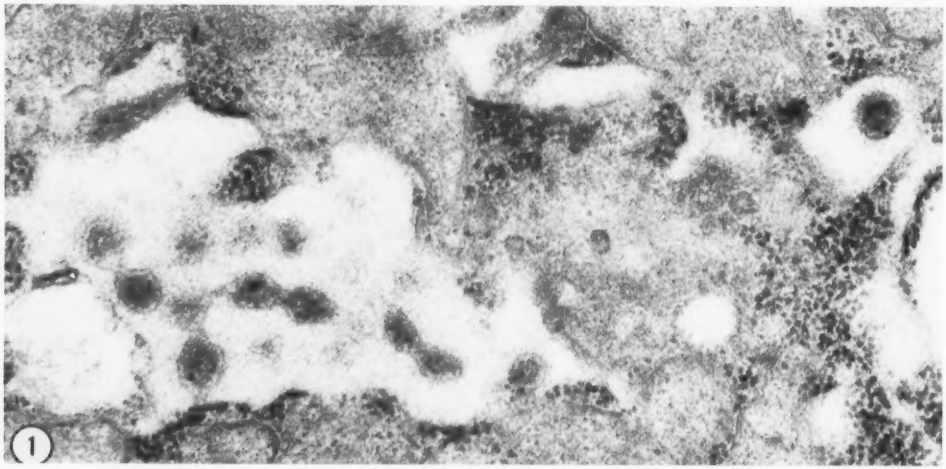

FIGURE 1.—Virus-like particles in rough endoplasmic reticulum of epithelial cells of a case of human PCa (× 40,000).

FIGURE 3.—Virus-like particles in the rough endoplasmic reticulum of epithelial cells of human PCa. Note peculiar filamentous or chainlike patterns and a budding particle (arrow) (× 40,000).

cles, frequently showing tubular, filamentous, or chainlike arrangement (fig 3).

IMMUNOLOGIC STUDIES

Information on the immunologic aspects of human cancer has been mounting through studies of a variety of human tumors including PCa (3–5). The present study was carried out in an attempt to determine whether PCa tissues carry antigens capable of eliciting tumor-associated antibodies, and whether antigens similar to those of oncogenic viruses are present in PCa and BPH tissues and are capable of eliciting antibodies in sera of patients with prostate neoplasia.

Both short-term and long-term cell cultures have been used in this study. Short-term cultures included seven cultures derived from PCa, two from BPH, and one from histologically normal prostate tissues. Long-term cultures comprised a cell line developed in our laboratory and derived from PCa tissue (cell line P-119, fig 6) and two cultures developed in our laboratory, one from prostatic tissue of an 18-week-old human fetus and one from the prostate of a 2-day-old infant. The tissue specimens were collected at autopsy under sterile conditions and kindly provided by Dr. Theodore I. Malinin, University of Miami School of Medicine, Florida (6). Culture MA-160 derived from prostate adenoma at passage 15 was kindly provided by Dr. Monroe M. Vincent, Microbiological Associates, Inc, Washington, DC.

HeLa and KB cultures were derived from human cervical carcinoma (CCa) and epidermoid carcinoma of the oropharynx, respectively, and obtained from the American Type Culture Collection Cell Repository, Rockville, Maryland. None of the cultures established or maintained in our laboratory was found by electron microscopy to contain mycoplasma or virus particles.

Human Sera

A total of 230 human sera were tested. They were obtained from donors of the following categories: 48 PCa patients; 23 BPH patients; ten CCa patients; 98 patients with other neoplasms (34 leukemia, six lymphoma, nine Hodgkin's disease, ten reticulum cell sarcoma, nine osteosarcoma, 15 soft tissue sarcoma, nine melanoma, one glioma, one carcinoma of the colon and urinary bladder, one thyroid carcinoma, and three polycythemia); and 55 apparently normal individuals (42 men, 13 women).

Mouse Prostatic Tissues

Normal prostatic tissues of 46 mice from ten different strains were examined for the presence of virus particles by electron microscopy. Prostatic tissue specimens of dorsal and/or ventral lobes were obtained from young (1–3 months) and old (8 months to 2 years) mice of high (C3H/Dm, RIII/Dm, and A/Dm) and low (BALB/c/Dm, C3Hf/Bi/Dm, and C3Hf/He/TEX) mammary cancer strains, and high (AKR/Dm) and low (CBA/J/Cr, SJL/J/Cr, and C57BL/6/TEX) leukemia strains. The tissues from the pros-

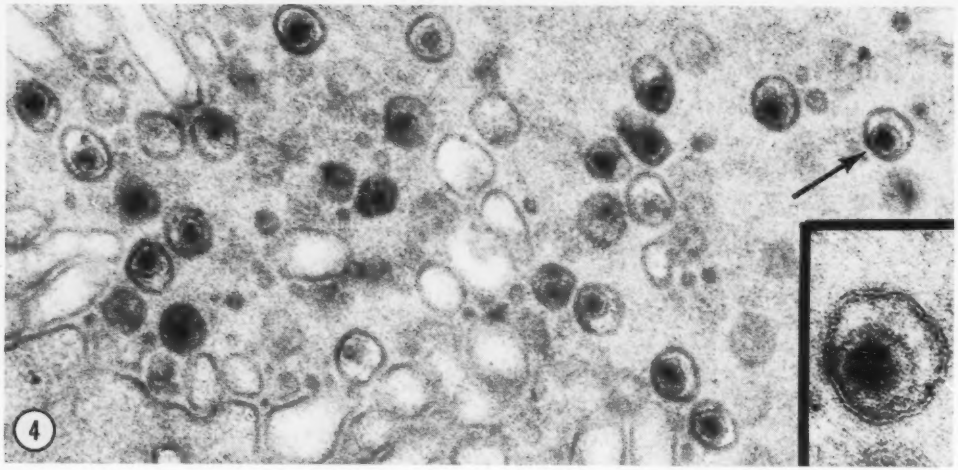

FIGURE 4.—Mature type B virus particles in glandular lumen of normal prostate of a 431-day-old A/Dm strain mouse (× 60,000). Insert: High magnification of 1 of the particles (arrow) (× 150,000).

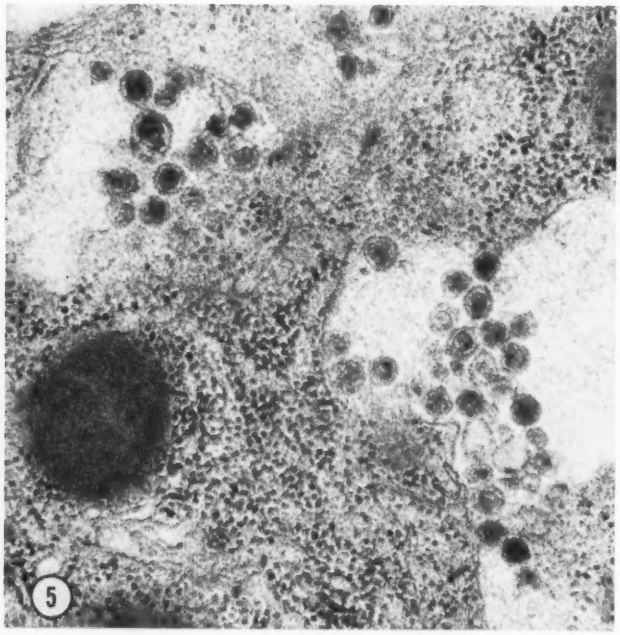

FIGURE 5.—Mature and immature type C virus particles in the intercellular space of normal prostate of a 385-day-old AKR/Dm strain mouse (× 40,000).

tate gland of each mouse were processed in the same manner as those of human prostatic tissues.

Type B virus particles and budding were found associated with intracytoplasmic type A virus particles in the prostate glands of four old (10–14 months) mice of three high strains (C3H/Dm, RIII/Dm, and A/Dm) and in the prostate of a low (BALB/c/Dm) mammary cancer strain mouse (fig 4) among the 46 mice of ten different strains examined. Type B virus particles have been found only in the glandular lumina of dorsal lobes of the old mice but not in the ventral lobes. The prostate glands of all four old mice which contained type B virus particles also contained a number of type C virus particles in different parts of the same glands.

Type C virus particles and budding (fig 5) have been observed in the dorsal and ventral lobes of the prostate of 25 young and old mice from all ten strains examined. Type C virus particles and budding have been found in the glandular lumina and intercellular spaces of epithelial cells. The normal prostate tissues of older mice contained larger numbers of type C virus particles than those of young mice.

It is of interest that in the prostate glands of all 46 mice from the ten different strains, varying numbers of intracisternal type A virus particles could be seen in the dorsal and ventral lobes of the prostate. These particles have always been found in the rough endoplasmic reticulum of glandular epithelial cells and infrequently also in the stromal cells of the prostate of mice of some strains.

Antivirus Sera

Goat antisera to interspecies cross-reactive viral protein or viral glycoprotein of several animal RNA tumor viruses were used in the present study. Anti-MuLV p30, anti-MuLV gp69/71, anti-FeLV p27, and anti-SSV p30 sera were kindly provided by Dr. J. Thomas August, Albert Einstein College of Medicine, Bronx, New York. Details of these antisera were published (7). Anti-SLV gp69/71 serum was prepared and kindly provided by Dr. Stephen J. Kennel, Scripps Clinic and Research Foundation, La Jolla, California, by a method previously published (8). Anti-MPMV p10/12 (identification No. 3S-39) and anti-MPMV p27 (identification No. 4S-517) were prepared using MPMV grown in human NC-37 cells at Huntington Research Center, Baltimore, Maryland, under The Virus Cancer Program of the National Cancer Institute, Bethesda, Maryland, and obtained through the courtesy of Dr. Jack Gruber of that Institute. Goat anti-FeLV-R serum was prepared by us as described previously (9). Rabbit anti-HSV-2 serum was prepared by using HSV-2 grown in human Hep-2 cells and kindly provided by Dr. Laure Aurelian, The Johns Hopkins University School of Medicine, Baltimore, Maryland (10). Anti-MPMV p10/12 and p27 sera were absorbed twice with lysates of NC37 cells. Anti-FeLV-R serum was absorbed twice before use with homogenates of pooled tissues of normal cats. The anti-HSV2 serum was absorbed twice with lysates of Hep-2 cells. All human sera and anti-virus sera were heated at 56°C for 30 minutes before use. For initial screening, human sera were tested at a 1:8 dilution. For titration, human sera and anti-virus sera were tested at twofold serial dilutions. The highest dilution of the serum giving positive reaction in repeated tests was considered as the fixed immunofluorescence (FIF) titer of the serum. A number of sera of PCa patients reacted positively with four short-term cultures of PCa (61%, 15%, 7%, and 4% respectively). None of the sera reacted with fetal and infant prostate cells. Sera of patients with BPH did not react with cells of any of these cultures.

Strikingly different results were observed in FIF tests with cells of long-term cultures such as P-119 prostate, HeLa, KB, and MA-160. Cells of P-119 line reacted positively with 91% of the sera of PCa patients (fig 7) and with 32% of the sera of patients with BPH. They also reacted positively with sera of PCa patients (63%) and sera of CCa patients (50%), and a low reactivity was achieved with normal donor sera (9%).

All the positive reactions observed were of the cytoplasmic type, often with strong perinuclear staining. Two of the tissue cultures in low passage derived from PCa reacted with autologous sera.

Unfortunately there were not enough BPH cells available for a systematic screening with the same batteries of human sera.

FIF tests have also been carried out to determine the presence of antigens of oncogenic animal RNA

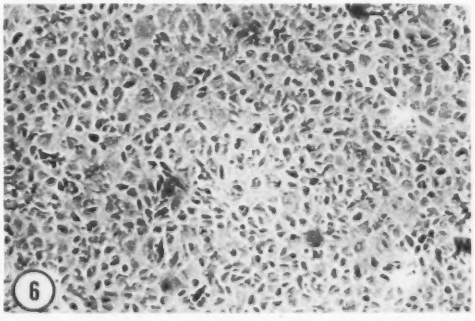

FIGURE 6.—Light microscopic appearance of cells of the P-119 cell line in passage 3 (× 82).

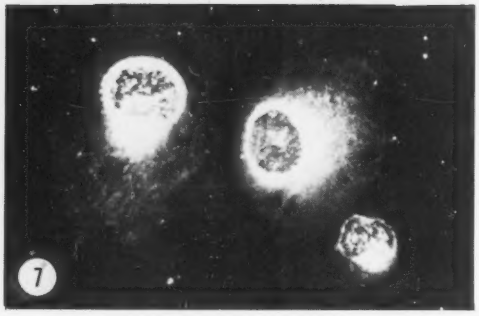

FIGURE 7.—Positive cytoplasmic fluorescence observed in FIF test of P-119 cells with serum of a patient with PCa (× 1325).

viruses and of herpesvirus type 2 in cells of some of the prostate tissue cultures. The P-119 cells gave a positive FIF reaction, although at low titers with antisera to interspecies antigens (p30) of murine, feline (fig 8), or simian type C viruses and gp69/71 of MuLV and Scripps LV but not with antisera to Mason-Pfizer monkey virus antigens (p27) or herpesvirus type 2 virus antigens. Cells of passage cultures derived from three PCa, two BPH, and two fetal human prostates were also tested, along with HeLa cells against goat antisera to p30 antigen of murine AKR and Rauscher leukemia viruses. Both antisera gave strong positive cytoplasmic reactions with PCa, BPH-derived cells, and HeLa cells. These sera gave a distinctly weaker reaction with fetal and infant prostate cells at identical dilutions (1:8). The significance and specificity of these positive reactions are currently being evaluated.

Immunoelectron Microscopy

Sera from patients with PCa and BPH and from normal donors were tested by the indirect immuno-

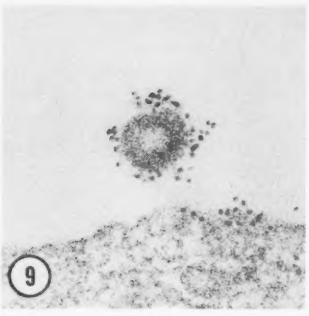

FIGURE 9.—Labeling of type C virus particles with ferritin by FIF-positive sera from a patient with PCa in AP-MSV cells (× 90,000).

ferritin and immunoperoxidase methods against type C virus-producing cells of Soehner-Dmochowski murine sarcoma virus (SD-MSV)-infected prostate tissue culture from a 42-day-old A/Dm strain mouse. For immunoelectron microscopy studies, a new technique of in situ embedding method of virus-producing cells grown in BEEM capsules was used as reported previously (11).

Prior to immunoelectron microscopy studies, sera from 60 cases of PCa, 24 cases of BPH, and 40 normal donors were tested by FIF against the cells of SD-MSV infected mouse prostate tissue culture (AP-MSV) cells. Among the sera tested, 23 cases of PCa (38.3%), six cases of BPH (25%), and 11 normal donors (27.5%) gave cytoplasmic fluorescence in AP-MSV cells. The remaining sera were negative.

All 11 cases positive by FIF (five patients with PCa, two patients with BPH, and four normal donors) gave ferritin labeling of type C virus particles and plasma membranes in AP-MSV cells and sometimes only of virus particles (fig 9). Sera of all FIF-

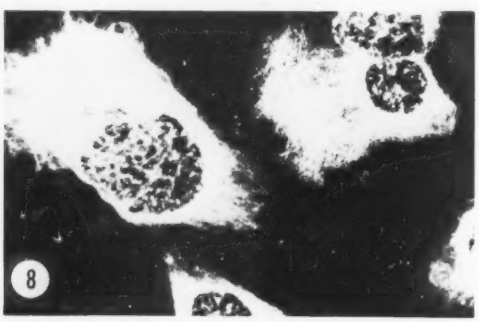

FIGURE 8.—Positive cytoplasmic fluorescence observed in FIF test of P-119 cells with goat antiserum to feline leukemia virus p30 (× 1325).

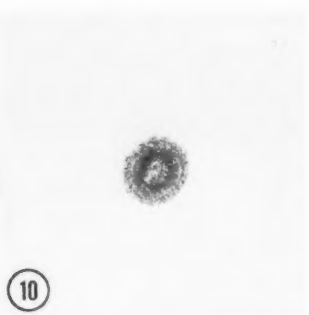

FIGURE 10.—Appearance of non-labeled type C virus particles by immunoferritin method by using FIF-negative serum from a patient with PCa in the same culture shown in fig 9 (× 90,000).

negative cases (four patients with PCa, three patients with BPH, and five normal donors) so far studied did not give ferritin labeling of type C virus particles (fig 10), but gave occasional labeling of plasma membranes. In the immunoperoxidase method, identical results were obtained.

BIOCHEMICAL STUDIES

Human prostate cell cultures and tissues have been examined for the presence of nucleic acids related to viral RNA genomes of known mammalian RNA tumor viruses. One avenue being explored is to examine established human prostate cell cultures for the release of particulate components containing high molecular weight (HMW) RNA which is a distinguishing feature of RNA tumor virus genomes. Any such HMW RNA species isolated and found to be present in sufficient amounts must be examined by molecular hybridization to determine whether they share nucleotide sequence homology with viral RNA genomes of representative mammalian RNA tumor viruses.

Two human prostate cell cultures so far have been analyzed for the release of HMW RNA species. They are the HFP-1 culture derived from a human fetal prostate and the P-165 culture derived from a case of BPH. These cell cultures were exposed to labeled uridine for periods of up to 2 weeks in efforts to increase the incorporation of radioactive precursor into possible HMW RNA species associated with particulate components released by cells. Fluid medium from the labeled cell cultures was harvested at weekly intervals, and particulate components were concentrated by differential centrifugation. After treating the components with 0.5% (wt/vol) sodium dodecyl sulfate to release RNA species, the sedimentation characteristics of these nucleic acids were examined by rate zonal centrifugation in sucrose (12). If an adequate amount of HMW RNA was detected, its structural integrity was measured by treating with pancreatic RNase A at high ionic strength to measure the strandedness or intracomplementarity of the molecules and by treating with alkali to establish the levels of DNA that could be present (13). For a given HMW RNA species to be considered satisfactory in terms of testing by molecular hybridization for sequence homology with viral RNA genomes of known mammalian RNA tumor viruses, at least 5000 cpm of acid-insoluble radioactivity had to be recovered from the gradient, and ribonuclease-resistant and alkali-resistant values of 15% or less had to be attained.

Both cell cultures released particulate components containing HMW RNA species. In the case of the HFP-1 cells, an HMW RNA with a sedimentation coefficient of 67S was noted, while with the P-165 cells the HMW RNA species had an observed sedimentation coefficient of 62S. Increasing the length of exposure of cells to radioactive uridine resulted in a marked increase in the amount of precursor incorporated into HMW RNA. Acid-insoluble radioactivity values for HFP-1 HMW RNA increased from 18,140 cpm at 1 week to 109,690 cpm at 2 weeks. Likewise, a similar finding was obtained with P-165 HMW RNA. In this case, an increase from 16,040 to 60,690 cpm was observed. Moreover, all of the HMW RNA species exhibited acceptable ribonuclease-resistant values of 0.7%–7.7% and alkali-resistant values to 1.4%–6.7%.

These results indicate that human prostate cell cultures release particulate components containing HMW RNA species and that these nucleic acids can be obtained in sufficient amounts and with adequate structural integrity to be considered satisfactory for use in testing for nucleotide sequence homology with viral RNA genomes of representative mammalian RNA tumor viruses. Molecular hybridization studies are now being conducted to examine this aspect.

Molecular hybridization studies, using radioactive labeled DNA probes of MSV-Ki (grown in rat kidney cells), MSV-M (grown in rat embryo cells), and simian sarcoma virus (grown in rat kidney cells) hybridized with cellular RNA extracted from different human prostate tissues have been employed to detect viral genetic information in PCa.

The data from molecular hybridization studies carried out under stringent annealing conditions indicate that cellular RNA from human prostate tissues share only a very few nucleotide sequences with MSV-M and MSV-Ki. Even though limited hybridization values were observed, cellular RNAs from some PCa and BPH tissues hybridized to higher levels with the viral DNA probes than cellular RNAs from normal prostate tissue.

DISCUSSION

There are very few reports on virus particles of human prostatic neoplasia (14,15). As already reported (2,16), our extensive ultrastructural studies on human PCa and BPH revealed the presence of intracisternal 150–200-nm virus-like particles and particles resembling type C virus particles. Further studies have demonstrated intracisternal 80–100-nm virus-like particles of similar appearance to that of hamster type H virus particles (17) in one case of PCa. The core of these intracisternal small virus-like particles differs from that observed in type H virus particles of tumors (17–19). Prostate tissue of this case of PCa contained intracisternal small and

large virus-like particles as reported in a case of human breast cancer (20). The intracisternal large virus-like particles are different from known oncornaviruses such as type A, B, and C virus particles (21). In our studies, virus-like particles have so far been detected in 13 of 49 cases of human prostatic neoplasia. They have been detected more frequently in PCa (12 of 37 cases) than in BPH (one of nine cases).

Type B virus particles in male mice are extremely rare (22,23). In our studies on normal prostates of mice, type B virus particles were present only in the prostates of old mice from high mammary cancer strains. These results suggest that prostate tissues of mice can be a potential source of horizontally transmitted mammary tumor virus.

Only one report of the presence of type C virus particles in spontaneous adenocarcinoma of the prostate gland of mice has been published (14). However, in our studies, type C virus particles have found in normal prostatic tissues of some mice of all the ten strains examined. It is of interest that prostate glands of mice of high leukemia strains contained type C virus particles in every cell type of prostatic tissues, even in lumina of capillaries.

The results of FIF tests add to the results previously published (16). The tests have shown that cells derived from PCa in vitro contained antigens recognized by antibodies present in sera of patients with PCa but rarely or not at all in sera of patients with BPH or of blood bank donors. The tumor-relatedness of these antibodies was indicated by their more efficient removal from serum by absorption with homogenates of PCa tissues than by absorption with other tissues such as BPH, CCa, or human embryo tissues. Similar tumor-related antibodies to prostate cancer cells have already been found in sera of PCa patients by others (5), but, as in the present experiments, the true nature of the antigens involved has not been elucidated so far. From absorption experiments with sheep red blood cells and guinea pig kidney powder, it would appear that antibodies in sera of PCa patients are essentially directed against Forssman-like antigens of PCa. However, this very important point requires further investigations.

Attempts to detect oncornavirus-related antigens in cells of PCa and BPH grown in vitro have shown, in FIF tests with specific antisera to these antigens, that the cells apparently carry antigens related to the interspecies p30 core protein (7) of murine, feline, and simian type C viruses. It is of interest that absorption of goat anti-AKR and Rauscher p30 sera with guinea pig kidney powder has failed to decrease the FIF titer of these antisera with PCa cells and HeLa cells. These results require further study. This particular immunologic approach has been used with success by previous investigators in other human tumor systems (24–26).

The results of the present tests with specific antisera to viral proteins are still too preliminary to allow any firm conclusions, but the data are encouraging enough to expect positive results in the near future.

Recently, attempts have been made to detect antibodies to type C virus particles or their constituents in human sera by other investigators (26,27). Our preliminary immunoelectron microscopy studies by immunoferritin and immunoperoxidase methods have demonstrated differences between FIF-positive and FIF-negative sera of patients with PCa and BPH and of normal donors, when tested against SD-MSV-infected prostatic tissue culture cells. There appears to be a correlation between FIF antibodies and those which label virus particles by ferritin or peroxidase. The nature of these antibodies is being further investigated.

The results of biochemical studies indicate that other viral probes will have to be employed in addition to those already used. Further, the effect of various conditions on hybridization procedures and on the amounts of nucleic acids extractable from prostate material will be explored.

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P-30 Antigen in Mouse Prostate [1]

Phyllis Kind [2,*]

C-type viruses and antigens associated with these viruses have been detected in the uterus and placenta of several mammals (1–7). In the uterus of the mouse, expression of a material that is antigenically related, if not identical, to the P-30 antigen of C-type viruses has been shown to be under hormonal control (8–10). However, hormonal control of this antigen in male tissues has not been reported. Since the prostate is an organ affected by hormonal levels, it was chosen as the tissue in which to study the hormonal control of P-30 antigen expression in the male. The purpose of this study was to discover if P-30 antigen can be detected in the mouse prostate and whether its expression is controlled by hormones.

MATERIALS AND METHODS

Mice

Male BALB/c mice were castrated or sham-castrated at 21–22 days of age.

Antiserum

Antiserum to the P-30 antigen was prepared by hyperimmunizing New Zealand white rabbits with purified P-30 antigen. Antigen, purified from banded Rauscher leukemia virus (RLV) by column chromatography on Sephadex G-100 followed by electrofocusing (11), was emulsified in complete Freund's adjuvant and a total of 15 μg was injected im and sc. Animals were bled 1–4 weeks after boosting sc with 5 μg of P-30 antigen dissolved in phosphate buffered saline, pH 7.2. Antisera reacted on Ouchterlony with purified antigen and with detergent-lysed RLV but not with preparations of avian leukemia viruses or with fetal calf serum.

Complement Fixation

Microcomplement fixation tests were performed by standard methods in microtiter plates. Block titrations were performed to detect the presence of antigens reacting with the known antiserum.

Rocket Technique

Weeke's (12) modification of Ressler's (13) rocket technique was used with the following major modifications. The electrophoresis buffer contained 15.85 g/liter of sodium diethylbarbiturate and was adjusted to pH 8.6 with approximately 18 ml/liter of 1 N HCl. This buffer was diluted with an equal volume of distilled water for inclusion in the agarose gel. The antiserum-agarose arena was 23-mm long and contained antiserum at final dilutions of 1:4 to 1:16. Antigen wells (2.5-mm diameter) were punched 2 mm inside the cathodic edge of the antiserum-agarose matrix. Each antigen well received 12 μl of antigen solution. Electrophoresis was conducted at a current of 10 microamperes per slide for 3–3 ½ hours. Slides were washed in normal saline, dried, and stained with Coomassie Brilliant Blue R.

Prostate Extracts

Nine weeks after castration, mice were killed by cervical dislocation. Prostatic tissue was removed and homogenized with 2 volumes of phosphate buffered saline, pH 7.2. Homogenates were centrifuged for 30 minutes at 2000 rpm and the supernatants were stored at −70°C. Because of the small amount of tissue, especially in castrated animals, tissue extracts from several mice were pooled.

RESULTS AND DISCUSSION

Extracts were assayed by the complement fixation test as a preliminary test to determine if P-30 antigen activity existed in the mouse prostate. Extracts from prostates of castrated mice reacted with specific antiserum as measured by complement fixation. Unfortunately, extracts of prostate from sham-castrated mice were anti-complementary. Thus this method does not appear to be useful for our purposes.

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The rocket immunoelectrophoresis technique was applied to the extracts of prostate because it can be used for both qualitative and quantitative analysis of antigens. We were able to detect as little as 80 ng of standard antigen with this method. P-30 antigen activity was found in the tissues of the castrated animals. Marginally detectable activity was found in the prostatic tissue of the sham-castrated controls. The amount of material in the prostate extracts of both groups was so small that it precluded quantitative measurements. Thus, accurate measurements could not be made.

Although P-30 antigen has been detected in mouse prostate by two immunologic techniques, complement fixation and rocket immunoelectrophoresis, neither technique is adequate for quantitative assays. Because of difficulties in labeling the antigen with ^{125}I, it has been necessary to use a large amount of labeled P-30 in radioimmunoassays and as yet the test is not sensitive enough to detect material in the prostatic tissue.

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Role of Oncornaviruses in Carcinoma of the Prostate[1]

Robert M. McCombs[2,3,*]

SUMMARY

The existence of oncornavirus genetic information in human prostatic tissue was studied by assaying tissue extracts for products of viral gene expression (ie, the p30 antigen). Tissue samples from patients with benign prostatic hyperplasia (BPH) or prostatic carcinoma (CaP) were assayed by the competetive radioimmunoassay. The competing antigens used were p30 proteins derived from the simian sarcoma virus type-1 (SSV-1) and the Rauscher murine leukemia virus (RLV). Of the 40 extracts tested, three of 20 extracts from BPH patients and one of 20 from CaP patients competed with the SSV-1 p30 antigen and only one of ten extracts from BPH patients competed with the RLV p30 antigen. The significance of these findings has yet to be established.

[Cancer Treat Rep 61:131–132, 1977]

The role of viruses as the etiologic agents of malignancy has been convincingly demonstrated in a number of animal systems (1,2). In recent years members of the herpesvirus and the oncornavirus groups have been implicated in malignancies of man (3–5). The present study was undertaken to determine whether or not members of the oncornavirus group are involved in carcinoma of the prostate (CaP) in man. The approach that has been chosen is to search prostatic specimens for products of viral gene expression, primarily, thus far, the p30 antigen, using both extracts of prostatic tissue and cell cultures derived from prostatic tissue that we are currently attempting to initiate.

MATERIALS AND METHODS

Collection of Prostatic Tissue

Prostatic tissue was obtained by Dr. Patrick Devine and his associates either by needle biopsy or transurethral prostatectomy from patients with benign prostatic hyperplasia (BPH) or CaP. The disease state of the tissue was determined histologically by the Pathology Department, Norfolk General Hospital. Normal tissue has been obtained at the time of autopsy.

In medium (Eagle's medium containing 5% fetal bovine serum) the tissue samples were transported to the laboratory as quickly as possible and were either processed immediately by a variety of methods for cell culture or frozen at –85°C for extraction and assay.

Assay of Tissue Extracts for p30 Antigen Activity

The frozen tissue was ground in a Dounce homogenizer in m/15 phosphate buffered saline (pH 7.2) and clarified by low-speed centrifugation. Thus far, 40 extracts of prostatic tissue, 20 from patients with BPH and 20 from patients with CaP, have been examined by the competitive radioimmunoassay for p30 antigen activity (6) using either the simian sarcoma virus type-1 (SSV-1) or the Rauscher murine leukemia virus (RLV) in collaboration with Dr. Gordon R. Dreesman (Baylor College of Medicine, Houston, Tex).

RESULTS

Human prostatic tissue specimens from 349 patients have been received by our laboratory. Of

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these, 227 have been characterized histologically and 122 are in the process of histologic examination. There were 52 specimens from patients with CaP, 173 from patients with BPH, and two from normal individuals.

The results of the competitive radioimmunoassay show (table 1) that there is competing activity in three of the 20 samples from patients with BPH and in one of the 20 samples from patients with CaP when using the SSV-1 p30 antigen; there is also activity in one of the ten samples from patients with BPH when using the RLV p30 antigen.

TABLE 1.—Assay of prostatic tissue for p30 antigen activity

	Samples reactive with—*	
Samples assayed	SSV-1-Ag	RLV-Ag
BPH	3/20	1/10
CaP	1/20	0/10

*No. of samples reactive/No. assayed.

DISCUSSION

The question of the nature of the competing antigen which has been detected in these samples has not been resolved. Due to the small quantities of each extract in terms of protein, it has not been possible to determine whether or not the reactive component is in the molecular weight range of 30,000.

The significance of the detection of an antigen that is able to compete with a known viral p30 antigen in 10% of the prostate extracts tested is an open question. It is not possible on the basis of these results to make a case either for or against oncornaviral involvement in human CaP inasmuch as the positive samples may not actually be due to viral gene products and the negative samples may contain viral antigens that are either below the level of detection of our assay or are prevented from competing due to some as yet undefined inhibitor.

Perhaps of equal importance would be to assay for p30 antigen activity in cell cultures derived from human prostatic tissue, both normal and abnormal, and to isolate a putative agent responsible for human malignancy. Toward this goal we have made efforts to initiate cultures of human prostatic tissue. Thus far, the parameters for the establishment of such cultures are under study and a number of parameters have been examined. When cell cultures become available either through our efforts or the efforts of others, the cultures will be examined for viral antigens and used in an effort to induce candidate human cancer viruses.

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Morphologic and Immunologic Studies of Human Prostatic Carcinoma[1]

Don D. Mickey,* Kenneth R. Stone, Marie P. Stone, and David F. Paulson[2]

SUMMARY

Benign and malignant prostatic tissue was removed in surgery and partitioned for (*a*) ultrastructural study, (*b*) tissue culture, and (*c*) immunochemical study. Fourteen malignant and 18 benign prostatic cancer specimens were examined by transmission electron microscopy (TEM) for the presence of viruses or virus-like particles. Viruses could not be identified with assurity in thin sections. Acinar cells of normal, benign prostatic hypertrophy (BPH), and neoplastic prostate tissue were examined in the scanning electron microscope and TEM and found to be extremely heterogenous in their surface morphologies. Three major types of surface morphologies were present: microvillous, ruffled, and bare. All three types of cells were present in normal, BPH, and neoplastic acini. A collagenase procedure was utilized to remove the stromal cells from glandular structures prior to in vitro cultivation. Partially purified extracts from 71 human urothelial tumors and 75 human urothelial nontumor tissues were used as competing antigens in competition radioimmunoassay in an effort to detect the presence of one of the structural components of type-C ribonucleic acid viruses, the p30 core protein. The urothelial tumors tested included 42 BPH specimens and 18 prostatic carcinoma specimens. Thirty-eight percent of the prostatic carcinoma tissues and 48% of the BPH tissues demonstrated the presence of a protein antigenically similar to the p30 core protein of an oncogenic RNA virus.

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Both morphologic and immunologic approaches have been used in the study of possible oncogenic viral relationships in human tumors and have provided considerable evidence that viruses are at least associated with such human tumors as leukemia, lymphoma, sarcoma, and breast carcinoma (1–3). Viral relationships have also been studied in human urogenital tumors (4,5). Other laboratories have investigated the possibility that C-type RNA viruses are involved or associated with prostatic carcinoma (6,7).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been used to study several human tissues including prostatic tissue. The principal objective in these studies has been to determine whether there are differences among the surface morphologies of normal, benign, and neoplastic cells. Both surgical tissues and in vitro-grown cells have been utilized in these studies (8–12).

MATERIALS AND METHODS

Acquisition and Fixation of Tissue

Prostate tissues were obtained from patients undergoing surgery for benign prostatic hypertrophy (BPH) or adenocarcinoma. The tissues were placed in cold medium in the operating room, brought to the tissue culture laboratory, and divided into portions for tissue culture growth and immunochemical and morphologic studies. Portions for morphologic studies utilizing SEM and TEM were processed as detailed by Stone et al (13). Tissues were fixed in 5% glutaraldehyde and 0.1 M

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sodium cacodylate buffer, pH 7.4, for 1 hour at room temperature and stored at 4°C (13). After fixation in glutaraldehyde the SEM tissues were rinsed in 0.1 M sodium cacodylate buffer, pH 7.4, containing 5% sucrose and post-fixed for 1 hour in 1% osmium tetroxide in sodium cacodylate buffer, pH 7.4. The tissues were dehydrated by washing in a graded series of ethanol and subjected to critical point drying, using Freon 113 as the intermediate fluid and liquid CO_2 as the transitional fluid. The dried specimens were mounted on SEM stubs, coated with platinum and carbon in a JEOL vacuum evaporator equipped with a rotary tilting stage, and examined in the ETEC "Autoscan" SEM operated at 10 or 20 KV. After fixation in 5% glutaraldehyde, TEM tissue specimens were washed and post-fixed in osmium tetroxide as described above for SEM specimens. TEM specimens were dehydrated in a graded series of ethanol and embedded in epoxy resin. Sections for light microscopy were cut at 0.5μ thickness on a Porter-Blum ultramicrotome and stained with toluidine blue. Thin sections for TEM were stained with 1% uranyl acetate and Sato's lead stain. All TEM specimens were examined in a Phillips 300 electron microscope.

Reagents for Tissue Culture

Growth medium was RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf or bull serum, 0.3% tryptose phosphate broth (Difco, Detroit, Mich), 0.0014% sodium bicarbonate, 0.005% streptomycin sulfate (Eli Lilly and Co, Indianapolis, Ind), 62.5 units/ml of Penicillin G (E.R. Squibb and Sons, Princeton, NJ), 0.5 μg/ml of Fungizone (Squibb), and 1 μg/ml of folic acid. Collagenase (type 1, CLS, Worthington Biochemical Corp, Freehold, NJ) was 1 mg/ml in growth medium filtered through a 0.45μ Nalgene filter.

Cell Culture

Prostatic tissues bathed in growth medium were dissected into pieces that were 1–3 mm in diameter with a scalpel. Small pieces were then incubated overnight at 37°C in 5% CO_2 with 5 ml of collagenase solution in a 60-mm Falcon Petri dish (14). The following morning the small prostatic chips were partially disrupted by enzymatic activity of crude collagenase. Gentle pipetting with a Pasteur pipet disrupted the tissue chips, such that the stromal cells were dispersed as single cells while the prostatic glands remained as large pieces, apparently existing as nearly complete glands. The glands were allowed to settle in a conical centrifuge tube for 15 minutes before the liquid covering them was removed by gentle suction, resulting in removal of the bulk of the fibroblasts. Five ml of fresh growth medium was added to the settled glands as a wash and the cells were allowed to resettle for 15 minutes. The medium was again removed and the glands were allowed to settle in a 60-mm Petri dish with 5 ml of growth medium.

Radioimmunoassay (RIA)

Viral reagents, antisera, tissue extraction procedures, ^{125}I labeling, and the RIA procedure, including the competition RIA, were utilized as described by Mickey et al (4).

RESULTS

TEM of Surgical Tissues

Fourteen malignant and 18 BPH tissue specimens were examined by TEM for the presence of viruses or virus-like particles. Although isolated virus-like particles having the appropriate structure of viruses were observed, viruses could not be identified with assurity in thin sections. Cellular inclusions identified as annulate lamellae were noted. These structures, having the same size and appropriate structure of viruses, are often mistakenly identified as viruses or virus-like particles.

SEM

Twenty-four BPH tissues and 21 adenocarcinoma and four normal prostate samples were examined in this study. Acinar cells of normal, BPH, and cancerous prostate tissues, when examined in the SEM, exhibit great variation in surface morphology. The cell surface morphologies may be classified as microvillous, ruffled, or bare; the microvillous cells comprise 90% of the acinar cell surfaces scanned. Short, stubby microvilli of 0.1μ–0.2μ in length and 0.05μ–0.1μ in diameter are present on villous acinar cells and range from a relatively few microvilli per cell to an extremely dense population. Other acinar cells are found which are covered with what is termed ruffles; they are measured to be 0.5μ–2.0μ in thickness. Others have described these ruffles as "ridge-like" or "microplicae." The number of ruffles per cell vary greatly. The bare cells of the acini are divided into two general types—cells which appear balloon-like, with no microprojections, and cells which appear identical to microvillous and ruffled cells in shape, but have an undulating surface rather than microvilli. Figure 1 demonstrates acinar cells of all three cell surface morphologies.

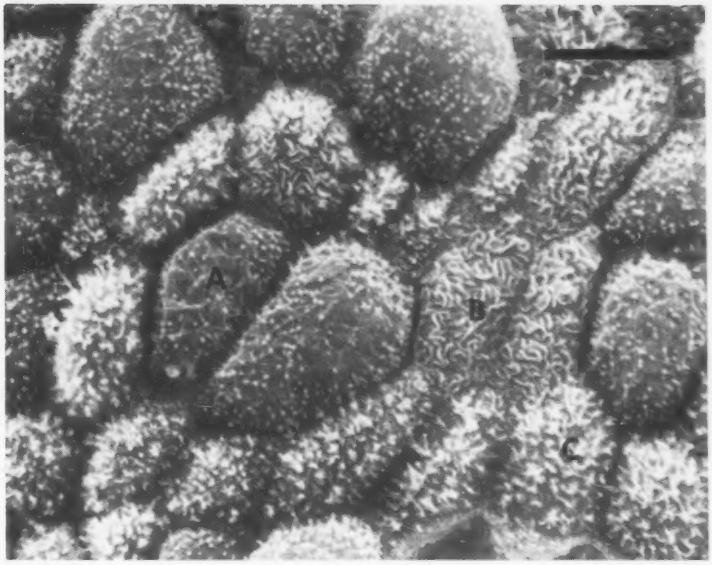

FIGURE 1.—SEM of acinar cells from prostatic adenocarcinoma. Bar = 5 μm; A = a bare cell with sparse, stubby microvilli; B = a cell with a ruffled surface; C = a cell densely covered with short microvilli. Strands of prostatic secretion may be seen attached to the bare cells (magnification, × 3500).

SEM of Tissue Culture Cells

Primary tissue cultures of glandular explants of BPH and adenocarcinoma prostate tissues were examined in the SEM. Cells spreading out onto the surface of the Petri dish displayed the same classes of surface morphologies as found in prostate acini observed from surgical specimens. The surface morphologies of tissue culture cells are representative of those found in surgical tissue, both in classification and percentages of the total population. The three types of acinar cell surface morphologies which are present when prostate tissues are examined in the SEM are also present in TEM of the same acinus (not shown).

Tissue Culture Growth Characteristics

Crude collagenase is effective for the isolation of viable prostate glandular cells. The glands are stripped of most of the supportive stroma without disruption of the gland structure itself. Even glandular material which has been in culture for 1 week or more still has the outside surface of a single gland configuration (14). Stromal cells are not present utilizing this method and only the epithelial cells are cultivated from the glandular structure. The characteristic junctions which appear between the epithelial cells comprising the glandular structure can be seen in the SEM studies (not shown). The tight junctions appear to be the same as those seen in TEM of the acini of prostate tissue fixed in section from surgery. In vitro, the glandular structures are viable and the outgrowth of prostatic epithelium begins growing radially from the glandular explant as a tightly packed epithelial monolayer. Cells grown in such a manner stain positively for acid phosphatase, indicating that the cells are derived from glandular epithelium of the prostate tissue.

Subcultivation of Tissue Culture Cells

Even with the utilization of the collagenase dispersion technique, subcultivation of prostatic cells into secondary culture is not consistent. Only very poor plating efficiency has been achieved using trypsin, EDTA, collagenase, Viokase, or combinations of this group. Cell monolayers appear to be quite difficult to disrupt into viable single cells. A single culture of prostatic adenocarcinoma derived

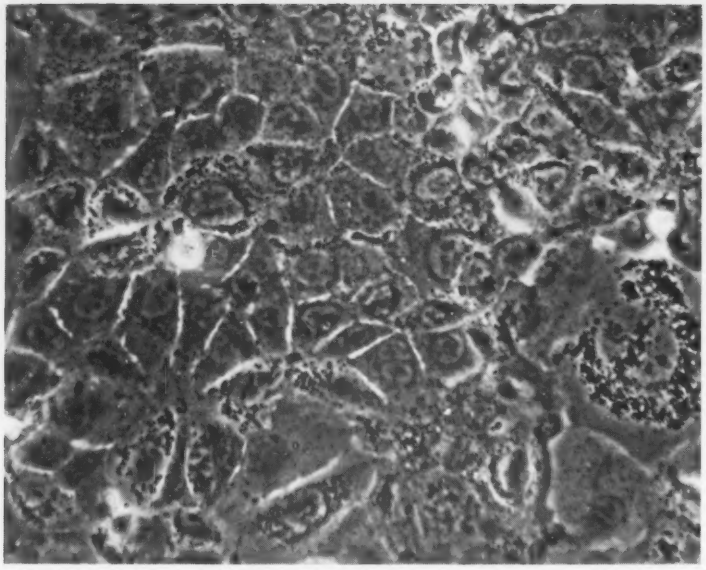

FIGURE 2.—Phase-contrast micrograph of prostatic epithelium in tissue culture (magnification, × 376).

from a metastasis to the brain has been maintained in culture after initiation via collagenase treatment for 11 months and has undergone 23 serial passages. The appearance of this cell in in vitro culture is shown in figure 2. This particular culture was also assayed for immunochemical demonstration of viral p30 antigen both from the original tumor specimen and from the tissue culture extracts; p30 antigenic activity was present in both extracts. TEM studies of these cells after in vitro passage have not yet been completed. Additional immunologic analyses of these cells have demonstrated that both primary and in vitro culture extracts produce an immunoprecipitin band when reacted in agarose with anti-FeLV p30 rabbit serum. In addition, when subjected to a two-stage SDS-PAGE immunoelectrophoretic technique designed to identify both antigenic activity and molecular weight of reacting proteins, extracts of these cells indicate an antigenic protein with a molecular weight near 30,000 daltons (15).

RIA of Tissues for Viral Proteins

Screening of tissue extracts in competition RIA was accomplished in a heterologous assay system in which the antibody used was anti-FeLV serum and the antigen used was ^{125}I-labeled MuLV p30 antigen. The details of the competition RIA are as described by Mickey et al (4). Table 1 shows the results of competition RIA of urothelial tissues. The tissues are catagorized into two groups, those that demonstrated antigen-positive tissue extracts and those that were antigen-negative in the heterologous RIA system. Of the 146 tissues assayed, 28% showed evidence of competition for p30 antigen. Thirty-eight percent of the prostatic adenocarcinoma tissue extracts were scored as positive as were 48% of the BPH tissue extracts. Positive extracts were not limited to tumor tissue and it is evident that competing protein antigenic activity was present as

TABLE 1.—Competition RIA of surgical tissue extracts using ^{125}I-labeled murine p30 antigen and feline p30 antiserum

	Competition		
	Present	Absent	Totals
Prostate			
Tumor	7	11	18
Nontumor	0	2	2
BPH	20	22	42
Kidney			
Tumor	3	18	21
Nontumor	1	20	21
Bladder			
Tumor	9	23	32
Nontumor	1	9	10
			146

frequently in benign urothelial tissues as it was in malignant urothelial tissue extracts.

DISCUSSION

Stone et al (13) previously reported that the acinar cells of neoplastic prostate are consistently more heterogenous in size and shape than the acinar cells of BPH tissues. This is an overstatement and a restriction must be made with regard to the differentiation of the neoplastic cell. Moderate to poorly differentiated carcinomas show much heterogeneity in acinar size and shape but a well-differentiated adenocarcinoma of the prostate does not. It is impossible to differentiate a normal, BPH, or well-differentiated carcinoma cell on the basis of size or shape.

Although several classes of surface morphologies exist in prostatic carcinoma cells, they do not seem to differ quantitatively or qualitatively in the normal, BPH, or well-differentiated carcinoma acini. Ruffled cells have been identified in kidney-collecting tubules (16), stratified squamous corneal epithelium (17), and human lymphocytes and monocytes (18). It has been suggested that these ruffles are actually collapsed microvilli. The bare cells of two types, those with undulating membranes and those with balloon-like structures, have been seen in SEM of prostatic epithelium. TEM sections of these same bare cells suggest that the balloon-like structures are artifacts due to improper fixation because they contain dispersed, watery cytoplasm. The bare cells with undulating membranes demonstrate the general ultrastructure of prostate cells.

One objective of the SEM study was to correlate the surface morphologies of SEM-observed cells from surgical tissue with the same cells cultured in vitro. It is demonstrated that the acinar cells in tissue culture retain their surface morphology as they spread onto the surface of the Petri dish. There is no definitive surface morphologic marker, however, which would indicate that these cells may not be of endothelial origin. The great heterogeneity of cell surface morphology in well-differentiated adenocarcinoma, BPH, and normal tissues prevents a correlation of cell surface structure in tissue pathology. It thus appears improbable that the surface morphology of a tissue-cultured prostate cell may be used to correlate that cell with the pathology of the original acinar cell.

The in vitro collagenase procedure seems to be an advantage in the in vitro culture of glandular epithelial cells from prostate and an aid in the separation of these acinar cells from supporting stroma. The source and purity of the collagenase itself must be considered because it appears that different lots of the enzyme are more effective than others for tissue disruption. Further, it appears that crude collagenase is more effective than a highly purified enzyme, suggesting that the real action of the preparation is the combined action of several enzymes such as proteases which act in concert with the collagenase itself.

An additional observation of the growth characteristics of prostatic acinar cells in in vitro culture from the collagenase plating method should be noted. Although initial outgrowth was rapid, once a limit of fast growth had occurred there was no substantial further spread of the cells across the dish even after several additional weeks of incubation. The reason for this limitation of cell growth is not clear, but could indicate a deficiency of a supportive matrix on the culture dish itself. Perhaps the stroma which could provide the necessary matrix for the continued growth of the acinar cells is necessary (14).

A discussion concerning the interpretation of evidence for proteins in prostatic tissue extracts which react with the p30 core proteins of oncogenic RNA viruses has been published elsewhere (4). It must be re-emphasized that, although the demonstration of antigenic cross-reactivities such as those reported here are consistent with the possibility that the prostatic tissues contain p30 antigen or p30 antigen-like proteins, additional biochemical evidence of protein similarity must be demonstrated. Observations and demonstrations of reverse transcriptase activity in these same cells would be desirable, as would TEM identification of C-type RNA viruses. Previous investigations in this laboratory on both reverse transcriptase activity and identification of C-type RNA virus particles within prostatic cells have yielded negative results. Many additional samples must be investigated before a conclusion can be reached.

Studies are incomplete on the tissue culture cells which demonstrate p30 antigenic activity. Extracts of these cells, both from the original surgical tissue and after collagenase in vitro culture, demonstrate a protein which reacts with anti-FeLV p30 rabbit serum. Two-dimensional SDS-PAGE immunoelectrophoresis analyses of these extracts indicates that a precipitin band is formed with anti-FeLV p30 rabbit serum and that the molecular weight of the reacting protein from this extract is near 30,000 daltons. The fact that this activity is present in in vitro cells provides the opportunity for the isolation of a protein clinically useful as a diagnostic agent.

The physical presence of virus or virus-like particles in either BPH or malignant prostatic tissue may be difficult to confirm due to extensive secretory activity of the prostatic epithelial cells. In

addition, the presence of a virus does not necessarily imply an etiologic relationship with the diagnosis of prostatic adenocarcinoma. It may simply indicate that there is passenger viral genetic material which has been incorporated into the host cell genome and the immunochemical assays are detecting the presence of this genomic material. The fact that this material can be demonstrated in BPH as well as in malignant tissues may indicate that the viral proteins do not have an etiologic role.

In conclusion, normal, BPH, and malignant prostatic tissues have been collected from surgical specimens and examined (*a*) ultrastructurally, using both SEM and TEM; (*b*) in an in vitro culture system for the relationship of morphologic features to the original tissue and for the subsequent testing and supply of material for immunochemical studies; and (*c*) by an immunochemical approach in a search for possible C-type RNA viruses. The present areas of concentration include: (*a*) rapid and proper fixation of surgically obtained prostate material, (*b*) the most desirable in vitro culture conditions for secondary and subsequent tissue culture growth of prostatic acinar epithelial cells, and (*c*) the further immunochemical characterization of what appears to be the presence of a structural protein of C-type oncogenic RNA viruses.

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Cytomegalovirus and Cancer of the Prostate: In Vitro Transformation of Human Cells [1]

L. Geder,* E. J. Sanford, T. J. Rohner, and F. Rapp [2,3]

SUMMARY

Urogenital tissue specimens were maintained in culture for 2 years. Epithelioid growth was enhanced with use of collagenase digestion rather than trypsinization. Twenty of 34 prostate cancer cell cultures survived more than ten in vitro passages, during which time four of 20 demonstrated epithelioid morphology. One epithelioid line (T-157) survived 32 in vitro passages. The cells demonstrated lack of contact inhibition in culture, were slightly positive in acid phosphatase tests, and reacted positively with cytomegalovirus (CMV)-immune sera in indirect immunofluorescence (IF) tests. These cells, which were proven to be of human male origin, failed to yield infectious virus and could be re-isolated from a nodule induced by the cells when injected sc into weanling athymic nude mice. The serum of the patient from which the tumor cells were derived demonstrated high CMV antibody titers and reacted with the virus-specific membrane and intracellular antigens of CMV-transformed human cells in IF tests. A CMV strain isolated from one of the normal prostate cell cultures established an in vitro long-term persistent infection of human embryo lung cells which resulted in the development of two transformed cell lines. The transformed cells possessed CMV antigenic markers and induced non-differentiated tumors when transplanted into athymic nude mice. The results constitute further evidence of the transforming capacity of CMV, and suggest that the virus may be oncogenic in its natural (human) host.

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Presently, adenocarcinoma of the prostate is the second most common cause of mortality from cancer in American men. The etiology of this cancer remains unknown. The significance of such epidemiologic factors as age, race, religion, and geography has been reported previously (1). Experimentally, neonatal hamster prostate and normal human prostate fibroblasts have been transformed in vitro with simian virus 40 and the adenovirus-simian virus 40 hybrid. The transformed neonatal hamster prostate cells that were transplanted into adult hamsters produced tumors which morphologically resembled human prostatic carcinoma (2,3).

Members of the herpesvirus group have been implicated as possible etiologic agents of certain human malignancies (4). The Epstein-Barr virus, for example, is associated with Burkitt's lymphoma and nasopharyngeal carcinoma (5). Herpes simplex virus type 2 (HSV-2) has been associated with cervical carcinoma (6–8). HSV-2 is also thought to cause malignancy in the genital tract in man, based on its venereal transmission and occasional presence in genital secretions (9). Human cytomegalovirus (CMV) has not yet been implicated in any particular type of human malignancy although certain properties of the virus resemble those of known oncogenic herpesviruses. It is evident, for example, that CMV may persist in human genitalia (10,11), can induce

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oncogenic transformation of hamster embryo fibroblasts (12), and stimulates host cell DNA and RNA synthesis. According to recent publications (13,14), CMV may have an etiologic role in human cervical neoplasia. We have reported (15) that cells from human prostate tissue which had been infected in vivo with CMV grew in vitro to passage levels higher than those routinely attained by normal cells. After a number of passages, virus was no longer detectable, although CMV-specific antigens and nucleic acid continued to be present in the cells.

This report describes the screening of prostate cell cultures for herpesvirus-specific markers and studies of in vitro transformation of human cells with a CMV strain (15) which had been isolated from normal human prostatic tissue. Our observations suggest the presence of CMV markers in some prostatic cancer cell lines and establish the in vitro transformation of human cells by the human prostatic CMV isolate.

MATERIALS AND METHODS

Cell Culture

Tissues removed at surgery were suspended in Dulbecco's medium and 10% fetal calf serum (FCS) containing 1 mg/ml of collagenase for 18 hours at 37°C. The medium was then replaced with fresh Dulbecco's medium and 10% FCS. Cell cultures surviving more than ten in vitro passages were maintained in Ham's F-10 medium with 20% FCS.

Induction of infectious virus by co-cultivation with human embryo lung (HEL) cells, by ultraviolet (UV) irradiation, and by pretreatment with iododeoxyuridine (IUdR) was carried out as described previously (15). An indirect immunofluorescence (IF) test was performed with human CMV convalescent sera following the method described in a previous report (15). In lymphocyte cytotoxicity tests, spleen cells from CMV-sensitized hamsters and Cx-90-3B, T-2 CMV tumor-bearing hamsters were used. The method has been described (15).

Animal Studies

Weanling athymic nude mice (NIH Swiss Webster, sixth back-cross generation) were obtained from Life Sciences (St. Petersburg, Fla). Cell sheets were trypsinized with a mixture of 0.5% trypsin and 0.02% versene. The cells were resuspended in medium and 4×10^7 cells were transplanted sc into the middle back of weanling 2–3-week-old athymic nude mice.

Tissue from animals was fixed in neutral buffered formalin, divided into 5μ paraffin sections, and stained with hematoxylin and eosin. Acid phosphatase tests were carried out on acetone-fixed cells stained with a mixture of naphthol AS-MX phosphate and fast Violet B for 30 minutes. After washing with water, the preparation was counter-stained with methylene blue.

RESULTS

In Vitro Cultivation of Cells From the Genitourinary Tract

Maitland-type fragment cultures were prepared in plastic tissue culture flasks from genitourinary tract tissue specimens obtained at surgery. A total of 155 cell cultures were prepared (table 1), of which

TABLE 1.—Results of cultivation of cells from normal and malignant tissues of the genitourinary tract (1974–76)*

Properties	Normal prostate	Benign prostatic hypertrophy	Cancer of the—			
			Prostate	Bladder	Kidney	Seminoma
Total No. of cell cultures	30	55	34	29	6	1
Cultures surviving 10–20 in vitro passages	4 F	8 F	7 F	2 F	0	0
Cultures surviving 20–30 in vitro passages	1 F	8 F	5 F/2 E	0	0	0
Cultures surviving > 30 in vitro passages	1 F	1 F	3 F/1 E	0	0	0
IF tests positive for CMV antigens	1 F	0	1 F/1 E	0	0	0
IF tests positive for HSV antigens	0	0	0	0	0	0
CMV strain isolated	1	0	0	0	0	0

*F = fibroblastoid cells; E = epithelioid cells.

TABLE 2.—Effect of collagenase treatment on epithelioid growth of prostate specimens

Method	Total No. of specimens	Cell cultures with epithelioid growth		Cell cultures with fibroblastoid growth	
		No.	%	No.	%
Collagenase treatment	16	10	62.5	6	37.5
Maitland cultures	21	8	38.6	13	61.4

34 cell cultures were of prostatic cancer origin. The primary outgrowth of the tissue fragments was generally a mixture of fibroblastoid and epithelioid cells. In most cultures, the fibroblasts gradually replaced the epithelioid elements before passage 10 was achieved. However, two of seven prostate cancer cell lines surviving > 20 in vitro passages and one of three prostate cancer cell lines surviving > 30 in vitro passages consisted of epithelioid cells. None of the cell lines of normal or benign prostate origin had epithelioid elements through > 10 in vitro passages, although one line of each group reached > 30 in vitro passages.

None of the bladder, kidney, or carcinoma cell lines has survived > 20 in vitro passages. A CMV strain was isolated from one of the normal prostatic cell lines which had been infected in vivo (15). Two of the prostate cancer cell lines (T-157 and T-173) reacted with CMV-specific human convalescent sera in indirect IF tests. Both cell lines grew through > 20–30 in vitro passages. Line T-157 consisted of epithelioid and short fibroblastoid cells up to passage level 32.

Enhancement of Epithelioid Outgrowth by Collagenase

Tissue fragments were incubated with 1-μg/ml collagenase in Dulbecco's medium and 10% FCS for 18 hours at 37°C, after which medium without collagenase was added. Attempts were made to remove the limited number of fibroblasts from the epithelioid outgrowth at a later phase of cultivation with trypsin. This method enhanced the establishment of pure epithelioid cell cultures from prostate tissue (table 2, fig 1). However, the average in vitro lifetime of the epithelioid cell culture was apparently not extended. Replacement of L-valine with D-valine in the medium (16) seemed to be toxic for both fibroblastoid and epithelioid elements in the culture.

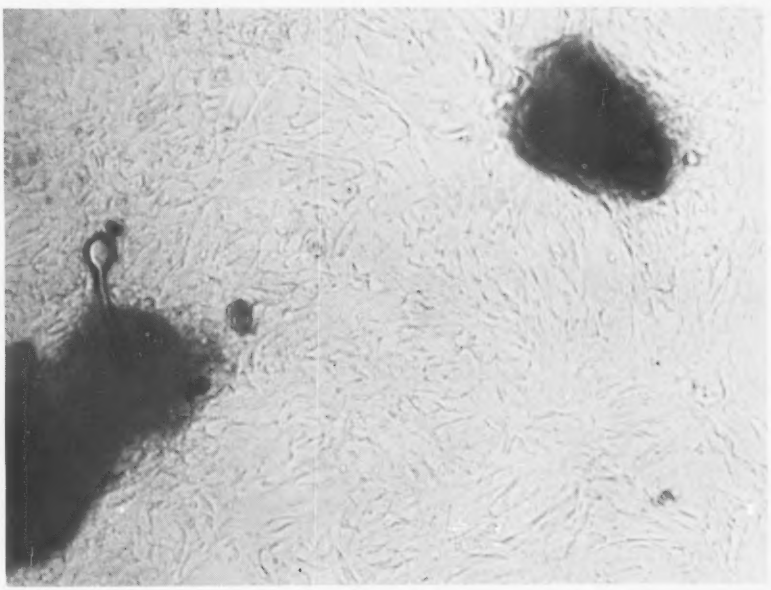

FIGURE 1.—Epithelioid growth of prostate cancer tissue in cell culture after collagenase treatment (× 500).

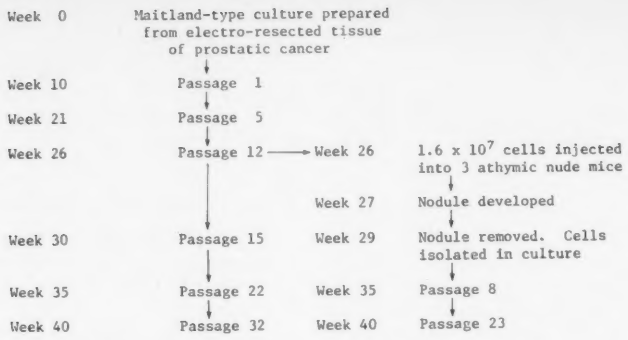

FIGURE 2.—History of prostate cancer cell line T-157.

Reactivity of CMV-Immune Sera

From 20 prostate cancer cell cultures surviving more than ten in vitro passages, two exceptional cell lines emerged. Line T-157 was derived from electro-resected tissue. The primary culture of this cell line exhibited very slow in vitro growth. Only scattered cells were attached to the surface of the culture flask for 12 weeks when the first sign of epithelioid and short fibroblastoid growth was observed. Following the loss of contact-inhibited growth during serial passages, the cells demonstrated an increase in nuclear size, tended to proliferate in multi-layered disarray, and continued to exhibit epithelioid morphology. The cells are at passage 32, 40 weeks after establishment (figs 2, 3A, and 3B). The T-157 cells were moderately positive in acid-phosphatase tests. Introduction of 2.5 μg/ml of testosterone served to temporarily improve cell growth. It was found that 40%–80% of the cells had membrane and intranuclear CMV antigens when tested with CMV-hyperimmune sera in indirect IF tests (table 3, fig 3C). No infectious virus could be recovered from these cells by inoculation of cell extracts onto HEL cultures or by co-cultivation with HEL cells. Irradiation with UV light (4 minutes at a distance of 200 mm, 42 ergs/second/mm^2) or pretreatment with IUdR (100 μg/ml in medium for 3 days) also failed to induce infectious virus.

Chromosome analysis of T-157 cells showed a normal human male karyotype (46 XY) with no chromosomal rearrangement. The patient's serum possessed a high anti-CMV antibody titer (1/256–1/512) in indirect IF tests. This serum reacted with the cell membrane-bound virus-specific antigens of in vitro CMV-transformed HEL cells (designated CMV-Mj-HEL-1 and CMV-Mj-HEL-2) and with the cells of tumors induced in athymic nude mice with CMV-transformed human cells (table 4).

Three athymic nude mice were hetero-trans-

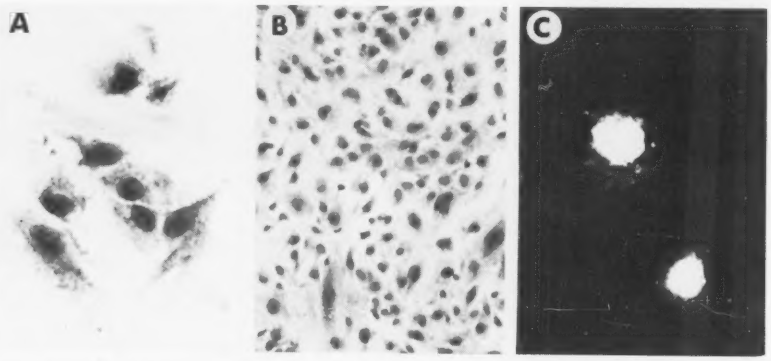

FIGURE 3.—In vitro culture of T-157 human prostate cancer cells (passage 9) stained with May-Grünwald-Giemsa (× 1250 [A], × 500 [B]). (C) Photograph of nuclear fluorescence detected in T-157 human prostate cancer cells (passage 9) after reaction with CMV-immune human convalescent serum and fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (× 1250).

TABLE 3.—CMV antigens in T-157 and T-173 human prostatic cancer cells detected with CMV-immune serum

	Cell membrane fluorescence						Intracellular antigens					
Serum	HEL control	HEL + CMV	Human bladder cancer	CMV-Mj-HEL-1* (%)	T-157 (%)	T-173 (%)	HEL control	HEL + CMV	Human bladder cancer	CMV-Mj-HEL-1* (%)	T-157 (%)	T-173 (%)
CMV-immune, human	–	+	–	60–80 +	40–80 +	10–15 +	–	+	–	80–100 + nuclear	60–80 + nuclear	10–15 + nuclear

*CMV-transformed human cell line in which foci of transformed cells appeared at passage 24 after infection.

TABLE 4.—Reactivity of serum from patient with prostatic cancer T-157 with the membrane of CMV-infected and CMV-transformed HEL in IF tests

Serum	Dilution	Cells				
		HEL control	Human bladder cancer	HEL + CMV	CMV-Mj-HEL-1*	CMV-Mj-HEL-2†
T-157 (pre-operation)	1/4	Negative	Negative	Positive titer 1:256 (1:512)	50%–100%+ titer: 1/8	41%–100%+
CMV-immune, human 9-B	1/10	Negative	Negative	Positive	80%–100%+	80%–100%+
HSV-2-immune, hamster	1/4	Negative	NT‡	Negative	Negative	Negative

*CMV-transformed human cell line in which foci of transformed cells appeared at passage 24 after infection.
†CMV-transformed human cell line in which foci of transformed cells appeared at passage 3 after infection.
‡Not tested.

planted with T-157 cells. Although no malignancy developed in any of the mice, the cells were re-isolated from a nodule which developed in one animal. Cells were re-cultured and have shown growth characteristics similar to those of the parental cell line at early in vitro passage levels. After a long initial stagnation, small islands of epithelioid and short fibroblastoid cells developed and formed piled-up foci. The cells are now in passage 23 and are growing faster than the parental line.

Cell line T-173 originated from a malignant perineal prostatectomy specimen. These cells experienced a much shorter latent growth phase and remained short and fibroblastoid throughout the in vitro passages. Only 10%–15% of these cells demonstrated cell membrane or intranuclear IF when tested with CMV-immune sera (table 3). No infectious virus could be rescued from these cells in passage 15 with the rescue methods used for T-157 cells. These cells grew to passage 35 in a 6-month period. No tumors or granulomas developed in 17 weanling athymic nude mice infected with 4×10^7 T-173 cells. The cells have a normal human male karyotype (46 XY) with no chromosomal rearrangement, and possess a very high mitotic index. No evidence of aneuploidy was demonstrated.

Human Cells Transformed by CMV

HEL cells were inoculated with a CMV strain isolated from normal prostatic tissue. Very low multiplicity of infection was used (0.001 plaque-forming

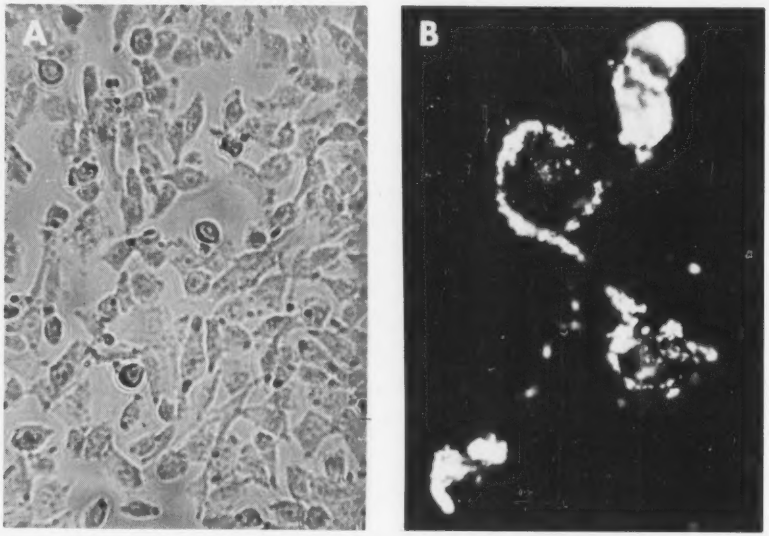

FIGURE 4.—(A) Photomicrograph of in vitro culture of CMV-Mj-HEL-2 cells (× 500). (B) Photomicrograph of membrane fluorescence detected on CMV-Mj-HEL-2, T-1 cells (passage 15) after reaction with CMV-specific human convalescent serum and fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (× 1250).

unit per cell). The virus-infected cultures were generally passed at 2-day intervals. Under these conditions, a persistent infection was established. Virus-induced cytopathology seldom spread beyond 50% of the cell sheet. At passage 23, the cells entered crisis. Shortly thereafter, foci of short fibroblastoid and epithelioid cells developed and formed a confluent cell sheet. These cells, designated CMV-Mj-HEL-1, were maintained in regular passage. They are now in passage 104.

Some months later, a subculture of CMV carrier HEL cells frozen at an earlier passage level was thawed and placed in culture. After 3 weeks, foci of transformed cells similar to the first transformed clone appeared and a second stable cell line, designated CMV-Mj-HEL-2, was developed.

The morphologically transformed cell lines were mixtures of short fibroblastoid and epithelioid cells (fig 4A). No infectious virus could be recovered by inoculation of cell extracts onto HEL cultures, or by co-cultivation with HEL cells. Irradiation with UV light or pretreatment with IUdR also failed to induce infectious virus.

CMV-specific membrane antigens were detected in these cells by indirect IF with different human convalescent sera (fig 4B). The antisera did not react with normal HEL cells. CMV-immune serum was adsorbed with CMV-infected HEL cells. This adsorption removed the reactivity of the immune serum for the CMV-Mj-HEL-1 and CMV-Mj-HEL-2 cell membranes. Adsorption with normal HEL cells did not affect reactivity. Hamster antiserum to HSV-2 or to PARA-7 tumor cells did not react with the CMV-Mj-HEL-1 and CMV-Mj-HEL-2 cell surfaces.

CMV-transformed HEL cells were specifically killed by spleen cells from hamsters immunized with CMV or from hamsters bearing tumors of CMV-transformed hamster cells. The survival of normal HEL cells and human bladder carcinoma cells was not significantly reduced. Thus, CMV-transformed human and hamster cells appear to have a common virus-associated membrane antigen(s). The sc inoculation of 2–4 $\times$ 10^7 cells of both transformed cell lines into athymic nude mice induced progressively growing tumors in 39 of 69 animals after a mean latent period of 16 days. No tumors developed in 85 mice transplanted with 4 $\times$ 10^7 CMV-Mj-P cells or in ten mice infected with 2 $\times$ 10^7 control HEL cells. Histologic studies of the tumors indicated that they were composed of small polygonal cells with large nuclei and scanty cytoplasm embedded in an abundant collagenous matrix. The cells were poorly differentiated but appeared to be of epithelial origin. Local invasion of adjacent structures was rare.

Normal and RNA virus-transformed cells generally respond to cytochalasin B treatment with one and occasionally two nuclear divisions, while DNA virus-transformed cells tend to become multinucleated. Treatment of CMV-Mj-HEL-1 and CMV-Mj-HEL-2 cells with cytochalasin B resulted in uncontrolled nuclear division, whereas nuclear division in HEL and bladder tumor cells remained controlled.

DISCUSSION

The question of whether viruses play a role in the etiology of prostatic cancer can be approached in different ways. The straightforward approach would be to detect viruses, virus markers (antigens), and virus genetic material in the prostatic cancer tissue or in in vitro cultured cells from the cancer. The prostate is the repository for the venereally transmitted genital HSV-2 (9) and CMV (10). Persistent asymptomatic viruria and viremia are detected in patients after CMV infection (11). CMV has also been isolated from the genital tract of several sexual contacts of virus carriers (17). The rate of CMV detection from the genitourinary tract would increase if the urine had no inactivating effect on the virus (18). Herpesvirus-like cytopathology (19) and virus particles have been observed in prostatic cell cultures (20,21), although no infectious virus was isolated. Herpesvirus antigens were detected in the nuclei of prostatic cancer cells (22) of primary explants. The presence of CMV in "normal" prostate tissue was confirmed by our recent report (15) of the isolation of a CMV strain from the tissue specimen of a 3-year-old donor. Our data confirm the presence of CMV antigenic markers in prostate cancer cells grown in vitro. The cells were positive for acid phosphatase but no infectious virus was isolated. However, the presence of CMV-associated antigen(s) in prostate cancer cells does not necessarily confirm the etiologic role of the virus in the oncogenic process. It is difficult to exclude the possibility of a chronic infection of the cells when an undetectable amount of the virus is excreted. Other indirect evidence for the oncogenicity of CMV is that the prostatic virus isolate can induce oncogenic transformation of human cells in vitro. In our experiments, the CMV isolate from a "normal" human prostate established a long-term persistent infection of HEL cells in vitro which resulted in the development of two CMV-transformed human cell lines. These transformed cells were oncogenic in weanling athymic nude mice.

In our preliminary study, sera from prostate cancer patients reacted two times more frequently in IF tests with the cytoplasmic membrane of CMV-transformed human cells than did the sera of the control group. Thus, the large-scale screening of

prostate cancer patients' sera against the cell membrane and intracellular/intranuclear antigens of CMV-transformed human cells and the determination of their cellular immune status against these cells may supply further evidence of the role of CMV in prostate tumor development. These combined approaches may well clarify the oncogenic potential of certain strains of CMV in its natural host and give more evidence for its role in the development of prostate cancer.

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Characteristics of Human Prostatic Cell Cultures [1]

M. Edward Kaighn [2,*]

SUMMARY

In preliminary experiments, the respective requirements of prostate-derived epithelial cells were compared with those of a human lung fibroblast line (WI38). Optimal levels for cysteine and glutamine were similar but not identical. The response of these two cell types to the macromolecular fraction of serum (dialyzed) was reciprocal. These results suggest that serum contains a dialyzable factor or factors necessary for the growth of prostatic epithelial cells.

[Cancer Treat Rep 61:147–151, 1977]

The potential usefulness of a cell culture system of prostatic epithelial cells is widely recognized (1,2). Whether this goal is possible, and by what means, is controversial (2,3). This situation is but a specific example of a more general one resulting from intensified national interest in the use of epithelial cell culture models in various aspects of cancer research. Thus, the cultured prostatic epithelial cell is viewed as a technical prerequisite for studies aimed at providing a rationale for the diagnosis, treatment, and eventual prevention of prostatic cancer.

In the past decade, epithelial and fibroblastic differentiated cells have been isolated from various sources (4). Because of our ignorance of the nutritional requirements of these cells, such successes were possible only by laborious empiric selection of undefined medium supplements such as serum. This approach, while sporadically successful, is far from satisfactory. Recently, significant progress has been made in improving the defined portion of the medium and by this means decreasing reliance on serum for the culture of WI38, a human diploid fibroblast line (5,6). Preliminary experiments with human prostate cells utilizing this approach will be reported.

MATERIALS AND METHODS

Prostatic tissue not necessary for routine pathologic examination was selected by the operating urologist at surgery. HP1–9 was derived (by Dr. P. R. Powell, Burlington, Vt) from a patient with benign prostatic hypertrophy; PC1 was derived (by Dr. John Horton, Albany Medical College, NY) from a bone marrow aspirate from a patient with metastatic prostatic carcinoma. WI38 was obtained from the American Type Culture Collection. Specimens were transported to the laboratory in chilled growth medium (ice bath) in F12KH containing 10% fetal bovine serum (FBS) and antibiotics. Primary cell suspensions were prepared by progressive dissociation and cloned as previously described (7). HP1–9 and PC1 lines used in this study were recovered from frozen stocks. The contents of an ampule (2–3 $\times 10^6$ cells) were diluted slowly with 10 volumes of chilled growth medium (F12KH + 10% FBS), gently sedimented by centrifugation (500 $\times$ *g*, 5 minutes), and inoculated at 10^6 cells/100-mm Petri dish containing 10 ml of medium.

For subculture, nearly confluent plates were selected. Medium was removed by aspiration and the monolayer was washed three times with HBM (Hepes buffered Moscona). CTC (0.1% collagenase, 0.1% trypsin, and 1.0% chicken serum, in HBM), pre-warmed to 36.5°C, was added (2 ml/100-mm plate). After 2–3 minutes, as much CTC as possible was removed without disturbing the cells. The dish was returned to the incubator and examined at intervals with a phase microscope. When the cells had rounded up from the surface of the dish, chilled medium (2 ml) was added. The plate was gently swirled and the cell suspension drawn in and out of a 14-gauge cannula affixed to a syringe. The cells were counted with a hemocytometer. Suitable dilutions were inoculated into 100-mm dishes (2–4 $\times 10^5$ cells/10 ml).

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[2] Pasadena Foundation for Medical Research, Calif.

**Reprint requests to:* Dr. M. Edward Kaighn, Pasadena Foundation for Medical Research, 99 N El Molino Ave, Pasadena, Calif 91101.

The standard medium used in this lab is F12K supplemented with selected 5%–20% FBS. Because of the instability of some components, F12K is prepared every 2 weeks from frozen stocks (8). Stock solutions are stored at −80°C. At this temperature they are stable for at least 1 year. Very recently we have added Hepes buffer (0.05 M, pH 7.6), reduced $NaHCO_3$ to 1.4×10^{-2} M, and decreased NaCl to 0.10 M. This is referred to as F12KH. Ham's latest medium developed for WI38 cells, MCDB104,[3] is prepared by an analogous protocol. All media used for primary cultures contain Penicillin G (100 units/ml) and Kanamycin (100 μg/ml). Once a cell line has been preserved in liquid nitrogen, antibiotics are deleted from future media for that cell line. All media, sera, and other solutions used for culture work are pressure filter-sterilized (0.22μ membrane).

Each lot of FBS is selected by a clonal assay for growth-promoting factors and low toxicity levels. Cell lines used for this purpose are CL9 (4), a normal rat liver epithelial line; WI38; and HP1–8, an epithelial human prostate line. The lot that gives the highest plating efficiency, the largest colonies, and the least evidence of toxicity is selected and stored frozen at −80°C until used. With most serum lots, 10%–17% FBS gives satisfactory results with human prostate cells both in primary culture and on subsequent passages.

[3]Ham RG. Personal communication.

FBS protein (FBSP) is prepared according to McKeehan et al (5). The serum is exhaustively dialyzed versus distilled water, lyophylized, and reconstituted with 0.13 M NaCl.

For evaluating various culture media, sera, and other parameters, a clonal growth assay was used. Cells prepared as described above were suspended in a convenient volume of culture medium lacking the materials being tested, and inoculated into experimental media at several levels so that a significant number of colonies were obtained (100–1000 cells in Corning No. 25010 Petri dishes [60 mm] containing 5.0 ml of test medium). The Petri dishes containing the experimental media were allowed to equilibrate in the incubator for at least 1 hour prior to inoculation. Immediately after inoculation, each dish was swirled gently to ensure uniform distribution of the cells over its surface. After all dishes had been inoculated, they were again swirled gently clockwise, counter-clockwise, then vertically and horizontally by moving the entire incubator tray that they were resting on. The dishes were then incubated for 2 weeks without medium change at 36.5°C ± 0.5°C in an atmosphere of 5% CO_2 maintained by automatic CO_2 control in air saturated with water vapor. At the end of the incubation period, the colonies were fixed with 10% formalin for 5 minutes, rinsed with distilled water, and then stained with 0.1% crystal violet for 10 minutes. Plating efficiency was determined by multiplying the number of colonies/dish × 100 and dividing by the number of cells inoculated.

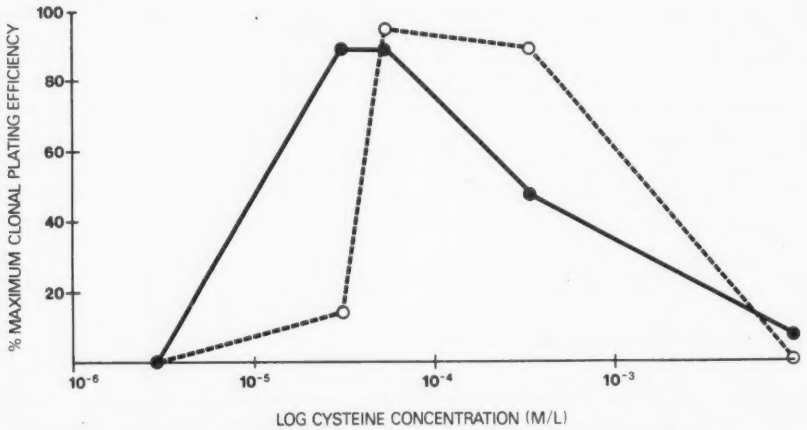

FIGURE 1.—Clonal growth response to cysteine in MCDB104 medium. ○ = WI38; ● = HP1–9.

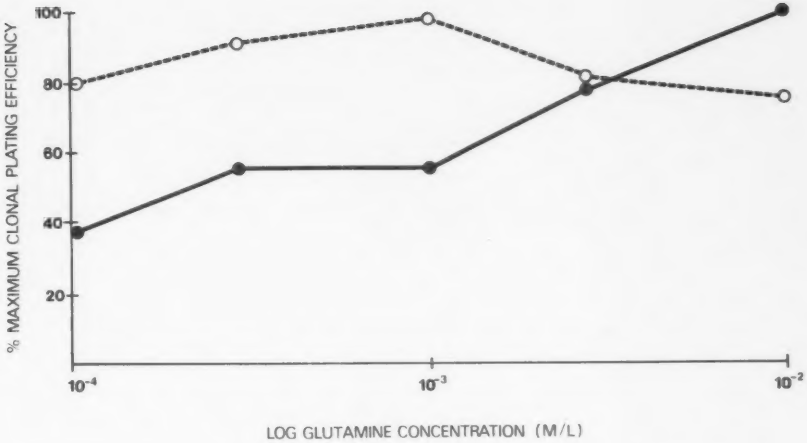

FIGURE 2.—Clonal growth response to glutamine in MCDB104 medium. ○ = WI38; ● HP1–9.

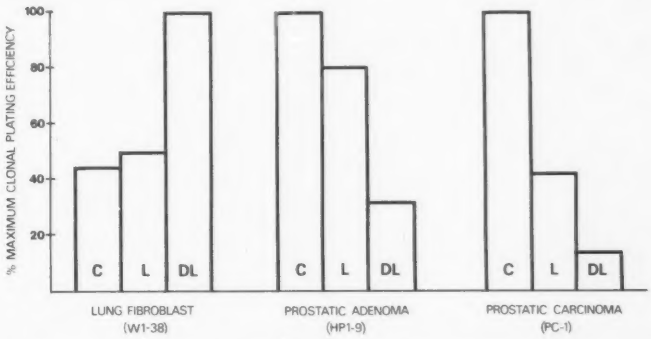

FIGURE 3.—Effect of serum dialysis on clonal growth of human cells in MCDB104 medium. C = untreated; L = lyophilized; D= dialyzed.

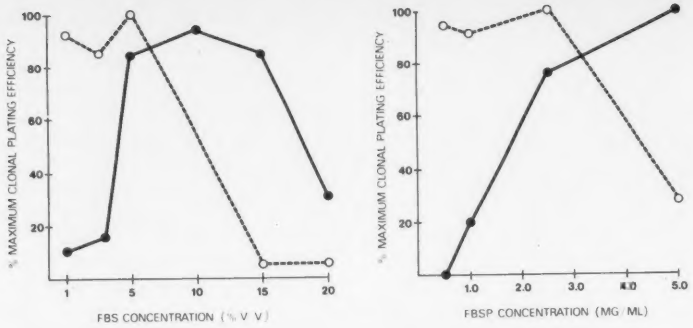

FIGURE 4.—Effect of dialysis on growth-promoting activity of FBS in MCDB104 medium. ○ = WI38; ● = HP1–9.

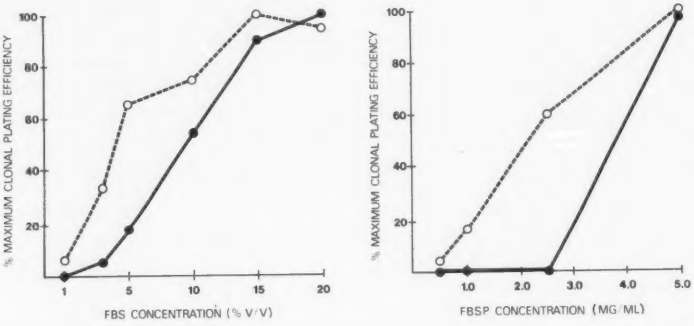

FIGURE 5.—Effect of dialysis on growth-promoting activity of FBS in F12KH medium. ○ = WI38; ● = HP1–9.

RESULTS

Nutritional Studies

Preliminary experiments were designed to determine whether clonal lines isolated from the human prostate had requirements for cysteine and glutamine similar to those of WI38 as reported by Ham and co-workers (6). The results indicate that WI38 requires and can tolerate somewhat higher levels of cysteine than HP1–9 (fig 1). With glutamine, no significant response was noted with WI38, whereas a definite increase in plating efficiency (2.5 ×) resulted from increasing the concentration from 10^{-4} to 10^{-2} M (fig 2).

Since these experiments were done with dialyzed lyophylized FBSP (5 mg/ml), the effect of this treatment was studied. Unexpectedly, dialysis affected prostatic epithelial cells and lung fibroblasts in a reciprocal way (fig 3). It improved the serum for WI38 cells while reducing its growth-promoting activity for prostate cells. When the growth-promoting activities of dialyzed (reconstituted FBSP) and untreated sera were compared, it became obvious that dialysis was removing some factor or factors necessary for prostatic cell growth (fig 4). WI38 was inhibited by higher levels of dialyzed serum. In untreated serum, there was marked growth inhibition for prostate cells > 15%. WI38 response was similar in both cases. FBS at 10% is about 4 mg/ml of FBSP with this serum lot.

The above experiments were all done with Ham's MCDB104 as a nutrient background. When our standard F12K was compared to MCDB104, the result was quite different (fig 5). With both cell lines, growth increased with increasing serum levels. However, with prostate cells there was no growth at all below about an equivalent of 6% dialyzed serum.

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Investigations on Prostate Adenocarcinomas in Rats [1]

Morris Pollard,* Chienkuo F. Chang, and Gary R. Burleson [2]

SUMMARY

Three transplantable prostate adenocarcinoma model systems in Lobund strain Wistar rats are described. The tumor cells have been cloned and propagated in vitro as monolayers of epithelial cells which reconstitute the same adenocarcinomas when inoculated into rats. All of the transplanted tumors spread from the sc implant site through lymphatic channels to the lungs, and thereafter to visceral organs and bones. Several applications of the tumor model systems are noted: they include chemotherapy, hormone therapy, and immunotherapy procedures aimed at revealing control regulations of tumor growth and metastasis.

[Cancer Treat Rep 61:153–156, 1977]

Spontaneous prostate tumors have been described in man, dogs, hamsters, and a Copenhagen rat (1). The etiologies of such neoplasms are unknown; however, the recorded incidence of prostate tumors in man usually rises with age. Attempts to induce prostate adenocarcinomas in animals have thus far failed. There is obvious need for an experimental tumor system of this type in animals that would reflect the disease in man.

Four years ago we encountered spontaneous adenocarcinomas of the prostate glands in nine aged germfree (GF) Lobund (Lob)-Wistar rats (2,3). Metastatic lesions were noted in the lungs of seven rats, and also in other visceral organs in five of the latter rats. Three of the rats with prostate tumors were positive for tartrate-inhibited serum acid phosphatase.

Prostate tumors of three rats with metastatic lesions were selected for animal passages. Tumor tissue was excised, minced with Medium 199, and inoculated sc into Lob-Wistar rats. They yielded tumor cell lines that repeatedly reproduced the original type of tumor in all implanted sites (4). Of unique significance was the manifestation of metastatic spread by all three tumor lines from the sc implanted site through ipsilateral lymphatic channels to the lungs. This occurred without special manipulations. No microbial agent has as yet been detected in the tumor cells (5). Only the Lob-Wistar rat strain (males and females) was susceptible to the tumor: Wistar rat strains from Charles River Laboratories, Harland Farms, and Glaxco were refractory. In the latter three rat strains, the implanted tumor cells produced a transient swelling which was involuted after the second week.

Eight of the autochthonous prostate tumors and their metastatic lesions were essentially the same: in seven of them, the prostate gland was replaced by tumor, and in one, a remnant of recognizable prostate tissue was detected (fig 1). The tumors were scirrhous and contained epithelial cells in disordered, multilayered, glandular arrangements separated by a prominent stroma of connective tissue and scattered areas of accumulated necrotic material. Mitotic figures were noted among the epithelial cells. Four of the tumors had prominent numbers of myelocytes dispersed among the other components of the tumors, which were reflected in enlarged spleens and in high myelocyte levels in the peripheral blood (2). Two transplantable tumor lines have been derived from them and designated tumor I and tumor III. Rats with tumor I differ from tumor III in having (*a*) myelocytes dispersed in the tumor and in the metastatic lesions, (*b*) enlarged spleens, and (*c*) some degree of myelocytic leukemoid reaction. In both tumors there was no evidence of host responses in the form of mononuclear cells in or near

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[3] Chang CF. Unpublished data, 1976.

FIGURE 1.—Adenocarcinoma in half of the prostate gland (arrow) from an aged (31 mos old) Lob-Wistar rat. Prostate tumor III was derived from this tumor. Hematoxylin and eosin stain, × 6.

FIGURE 3.—Rat showing metastatic spread of prostate I tumor cells from hind footpad through popliteal, inguinal, lumbar, and renal lymph nodes which had been inoculated into the footpad to the lungs. Note erosion of the foot by the tumor cells.

the tumors, nor could immunogenicity be demonstrable with them (4).[3] With both tumor lines, small metastatic tumors appear sequentially in the cortices of ipsilateral draining lymph nodes, in which the tumor mass expands to encompass the entire node. The metastatic spread of cells occurs within 10 days after sc implantation of tumor cells into the hind footpad. The transplanted tumors resemble the tumor from which they had been derived: they are scirrhous and consist of epithelial cells in a prominent matrix of connective tissue, plus variable areas of necrosis. The tumors are not encapsulated, nor do they have mononuclear cells in or around them. Within 3 weeks, small discrete tumor foci appear on and in the lungs. The tumor foci expand and eventually coalesce. In the early stages of lung involvement, the discrete round focal tumors on the surfaces of the lungs (fig 2) can be counted with the aid of a duoloupe. This information is useful in assessment of rates and extents of metastasis. In rats that survive beyond 60 days, tumor foci have appeared in the kidneys, in the heart, and in the bones. The site into which the tumor cells had been implanted becomes ulcerated and necrotic, to the extent that the entire foot may be eroded (fig 3).

A third transplantable tumor line (tumor II) was derived from a rat with a very large prostate tumor (34 g) which differed from the other tumors. It was soft and solid and contained homogeneous epithelial cells closely packed in nests which were separated by small thin strands of connective tissue stroma (4). This tumor spreads from the sc implant site through draining lymph nodes to the lungs, but the metastatic lesions differ from those of tumors I and III: prostate tumor II develops into very large masses (up to 60 g) at the implant site. Individual lymph nodes reach weights of 4.5 g (control lymph nodes = 0.06 g), and the expanding lesions in the lymph nodes and lungs are diffuse, not focal. Tumors appear in the contralateral lymph nodes, in the liver, and in the kidneys in rats that survive beyond 2 months.

Samples of tumors I and III were excised aseptically from the autochthonous tumors, minced in MEM + 20% fetal calf serum, and incubated in prescription bottles at 37°C. Epithelial cells and connective tissue cells grew from the explants. These were subsequently cloned and propagated in pure culture cell monolayers, and cells representing each clone were then inoculated individually into weanling Lob-Wistar rats. As few as 10^3 cells of tumor I and ten cells of tumor III scraped from the wall of the cell culture bottle reproduced the same

FIGURE 2.—Lung lobe from rat with focal metastatic tumors of prostate adenocarcinoma compared with control tumor-free lung lobe on the right. The lung was inflated and fixed in Bouin's solution and stored in 70% ethanol.

type of metastasizing tumors in the rats, whereas 10^6 of the connective tissue cells derived from both tumor lines were negative.[4] The epithelial cells stained positive for tartrate-inhibited acid phosphatase for four passages, and then this staining property turned negative. These tumor cells were negative for microbial flora when examined by culture for bacteria, by electron microscopy, and in tissue culture preparations for viruses. The cultures of epithelial cells have now been subpassaged > 70 times and their tumorigenicity is unchanged from that of the animal passage series. In vitro doubling times for prostate tumor I and tumor III cells are 22 and 18 hours respectively. The cells have been restored to activity from the frozen state. Examinations of these cells for prolactin receptors have been negative.

Tumor II does not propagate in vitro as readily as tumors I and III, but the tumor cells have been preserved frozen.

The actual value of tumor model systems should be assessed through their use in biomedical inquiries, which at the same time would help in further characterization of the tumors. The three prostate tumor systems described above are unique as model systems because of several attributes: they are of prostate origin, they are agnogenic, and they are adenocarcinomas without demonstrable immunogenic properties and without demonstrable microflora. They propagate in vivo only in the rat strain of origin. They propagate in vitro as monolayers of cells which reconstitute the tumor of origin when transplanted to Lob-Wistar rats. The tumors metastasize from the sc implant site through lymphatic channels to the lungs and thereafter to visceral organs. These are not acquired characteristics. They were observed in the rats from which the tumors had been derived.

On the basis of the unique attributes noted above, the prostate tumor model systems provide a methodology for investigations on several facets of the cancer problem: they have been and are being used for studies on therapeutic effects of chemical, antigenic, and hormone agents, and on factors that regulate the phenomenon of metastasis. In regard to the latter phenomenon, current inquiries seek to determine if metastasis is determined by a unique characteristic of the tumor cell or by a host regulatory mechanism (immunologic or metabolic).

The following protocol was developed for experimental applications of the prostate tumors:

Groups of weanling Lob-Wistar rats (males and females) were inoculated with tumor mince or tissue culture-propagated cells sc in the dorso-lumbar region or into the right hind footpad. They were further divided into groups for untreated controls and for treatments at Day 0 and/or at 7-day intervals thereafter. In the case of agents that cause immunosuppressive effects, the experiments were conducted in tumor-bearing GF rats. At intervals after onset (30, 60, and 90 days) groups of the rats were anesthetized, exsanguinated, and killed for examinations. The lungs were inflated with Bouin's fixative. Tissues (primary tumor, lymph nodes, and visceral organs) were weighed and samples thereof were fixed in Bouin's and examined as stained histologic preparations. The tumor foci in the lungs, appearing white on a yellow background (fig 2), were counted with the aid of a duoloupe and recorded. Data from treated and untreated animals were compared and interpreted.

Since rats with transplanted tumor II do not develop distinct lung lesions, their right and left homologous lymph nodes and their primary tumors were weighed and compared with weights of the same organs in untreated tumor-bearing control rats.

1. The three prostate tumors have been exquisitely sensitive to cyclophosphamide (CPA) if administered to rats at intervals of 7 days, soon after inoculation of tumor cells. Delayed onset of treatments resulted in the appearance of some CPA-resistant tumors. Cures, under all circumstances, were rare because tumors reappeared after cessation of CPA treatments (6).

2. Groups of tumor-bearing rats were treated with diethylstilbestrol (DES) (0.5 mg im daily or twice weekly). After 5 weeks, rats with tumor I showed some equivocal reductions in lung lesions and in weights of tumors compared to the untreated control rats. However, DES treatments of rats with tumor II resulted in very large primary tumors (average, 42 g) compared with tumors in untreated rats (average, 21.6 g). Thus, the two tumor types differed in sensitivity to DES (3).

3. Groups of rats were inoculated sc with prostate tumor I cells, and half of them were inoculated simultaneously and at weekly intervals thereafter with 1.4 mg of killed *Corynebacterium parvum* iv. The *C. parvum*-inoculated rats had fewer lung tumors (average, 1.3 per lung) than the untreated control rats (average, 13 per lung). The sizes of the primary sc tumors were essentially the same in both groups of rats. From this trial, it appears that *C. parvum* inoculations interfered with the rate and extent of metastasis.

4. At intervals after sc inoculation of tumor I cells, groups of the rats were anesthetized for 3 minutes by inhalation of chloroform or halothane. The anes-

[4]See footnote 3.

thesia-treated rats developed more lung metastases than the untreated control rats.

5. Tumor-bearing rats were subjected to whole-body doses of 400 rads of X-irradiation. This accelerated the rate and extent of metastatic spread of tumor cells to the lungs compared with the effect(s) in untreated control rats.

Since all tumor-bearing rats develop metastatic lesions in their lymph nodes and lungs, the experimentally derived results are clearly defined. This tumor model system should be a valuable experimental instrument for analysis of several significant facets of the cancer problem.

We do not know the etiology of these prostate tumors. We have not succeeded in inducing prostate carcinomas by experimental procedures; however, the episodic nature of the nine "spontaneous" cases suggests that the rats had been exposed to a "carcinogenic factor" in the course of their lifespan. Since this occurred in axenic rats, we may exclude the role of microflora in the carcinogenic process. Possibly the tumors appeared in the GF rats because they lived longer than their conventional counterpart rats, and the GF status had no role other than this in the development of the prostate tumors.

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Separation and Characterization of Epithelial Cells From Prostates and Prostatic Carcinomas: A Review[1]

Thomas G. Pretlow II,* Michael G. Brattain, and Jeffrey I. Kreisberg[2]

SUMMARY

Our investigation of normal, hyperplastic, and neoplastic prostatic tissue during the past 2½ years has produced several findings which have been published or accepted for publication. (*a*) Cells from hamster prostates with intense histochemically demonstrable acid phosphatase activity (HDAP) after fixation with formaldehyde which we believe to be epithelial cells can be obtained in 97.2% ± 0.8% purity by velocity sedimentation in a previously described isokinetic density gradient; (*b*) similarly, cells with HDAP, many of which contain lipofuscin granules, can be obtained as 81.0% ± 12.2% of nucleated cells from hyperplastic human prostates and as 86.4% ± 9.4% of nucleated cells from human prostatic carcinomas; (*c*) more cells were obtained from human hyperplastic prostates and prostates with prostatic carcinoma per gram of tissue with the aid of Pronase than were obtained with trypsin, collagenase, or mechanical methods; (*d*) more cells per gram of tissue were obtained from surgically removed prostates than from prostates obtained at even very rapid autopsies, and a much larger proportion of the cells from surgically removed prostates were viable as assessed both by dye exclusion and by plating efficiency; (*e*) none of several substrates and inhibitors which we tested were highly specific for acid phosphatase from purified prostatic epithelial cells compared with several other kinds of purified human cells; and (*f*) purified hamster prostatic epithelial cells incorporate large amounts of tritiated thymidine in 72-hour cultures.

[Cancer Treat Rep 61:157–160, 1977]

Normal prostates, hyperplastic prostates, and prostates with carcinoma contain many different kinds of cells in highly variable proportions. Normal interactions between epithelial cells, muscle cells, fibroblasts, vessel endothelial cells, and blood cells in the prostate are incompletely understood. These relationships are even less well understood in prostates with carcinoma. Our long-term interest has been the characterization of malignant epithelial cells from human carcinomas. We hope to be able to define qualitative or large quantitative differences between malignant epithelial cells and their normal, ancestral precursors which might be able to be exploited in the design of selective methods for killing the malignant cells in carcinomas without killing or injuring normal cells which are necessary to the patient's vital functions. In this report, we shall outline the progress which we have made in approaching this long-term interest particularly as applied to prostatic carcinoma. Because we believed that the availability of single kinds of cells would facilitate the chemical and immunologic characterization of malignant epithelial cells from prostatic carcinomas and prostates without carcinoma, our first task was to develop methods for the purification of these cells. Like the purification of single kinds of cells from other kinds of tissues (1), the purification of single kinds of cells from prostates required (*a*) that we evaluate methods for obtaining the cells in suspension, and (*b*) that we test the available methods for the purification of single kinds of cells from those suspensions. After finding satisfactory solutions to these two problems, we might approach the characterization of the purified cells.

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Methods for Obtaining Cells in Suspension

The selection of methods (1) for obtaining cells in suspension is of critical importance since the destruction or alteration of cells during the process of obtaining them in suspension may alter the extent to which they are truly representative of the cells from the organ of interest. We (2) have discussed this problem in detail previously and shall limit our discussion here to a statement that we tested the suitability of 0.25% trypsin, 0.1% Pronase, 0.05% collagenase, and purely mechanical methods for obtaining cells in suspension from prostates and prostatic carcinomas. For hamster prostate, 0.25% trypsin gave the largest number of viable cells per gram of tissue (3). For human, hyperplastic prostates (4) and prostates with carcinoma (5), the largest number of viable cells per gram of tissue was obtained with 0.1% Pronase. As previously reviewed (1), different batches of many proteolytic enzymes may vary considerably in their chemical composition and in their suitability for work with specific tissues. We have found particularly large differences among different batches of trypsin (6). We have found that the best yield of cells from human prostates and prostatic carcinomas is obtained if the tissues are placed in ice-cold tissue culture medium immediately after resection. As we reported in an earlier study of 18 prostates (4), larger yields of viable cells were obtained from surgically removed prostates than from prostates obtained from autopsies even when autopsies were performed within 1½ hours of death; this principle has been confirmed in our studies of numerous prostates since the first 18 which we reported (4). More importantly, we have found that cells obtained from surgically resected prostates have greater abilities to grow in culture than cells from prostates obtained at autopsy. Studies which are still in progress suggest that important biochemical changes have occurred in prostates which have not been cooled rapidly after their blood supplies were interrupted.

Purification of Cells From Prostates and Prostatic Carcinomas

We have evaluated isopycnic and velocity sedimentation as methods for the purification of prostatic epithelial cells. Isopycnic sedimentation was not effective. Velocity sedimentation in a previously described isokinetic gradient (7) has resulted in the concentration of cells with intense histochemically demonstrable acid phosphatase activity (HDAP) from hamster prostates, hyperplastic human prostates, and human prostates with carcinoma. In the purest fractions from many experiments which have been reported in detail previously, these cells were obtained as 97.2% ± 0.8% of nucleated cells from hamster prostates (3), 86.4% ± 9.4% of nucleated cells from human prostates with prostatic carcinoma (5), and 81.0% ± 12.2% of nucleated cells from hyperplastic prostates (4). We hope that the sequential combination of sedimentation in an isokinetic gradient and other methods for the purification of cells may be useful in the further purification of cells from human prostates. The several other kinds of techniques which are available for the separation of cells have been recently reviewed (1).

Identification of Purified Cells

One of the most difficult problems in cell separation is the identification of particular kinds of cells in suspension. Many of the cells which we have purified have been sufficiently distinctive cytologically so that identification was not a problem; eg, megakaryocytes (8) are multinucleated; parotid epithelial cells (9) have a very distinctive morphology which permits their unequivocal identification following purification; Reed-Sternberg cells (10) are easily identified; and the purified cells from the tumor of Hodgkin's disease which contain the tumor-associated antigens described by Order et al (11–13) are adherent to the Reed-Sternberg cells both in tissue sections and after purification (2). In the case of prostatic epithelial cells, the intense HDAP is very helpful; however, it must be remembered that other kinds of cells contain acid phosphatase, and it would be helpful to use other markers. Stonington et al (14) have reported that "... acid phosphatase of the prostatic epithelial cell is not inhibited by immersion in 10 per cent formalin up to 24 hours, whereas all other acid phosphatases are destroyed by formalin." We had also observed in frozen sections that formalin fixation made our stain more specific for prostatic epithelial cells and, for that reason, we used formalin with 1% calcium chloride for the staining of our purified hamster epithelial cells which we reported in abstract form (15) in 1974 and as an article in January 1975 (3). We were very pleased to see the very thorough documentation of the specificity of formaldehyde fixation by Stonington et al (14) and only wished that we had made the effort to perform and report our studies of formaldehyde fixation with the same care and detail as Stonington and his co-workers. His excellent, detailed study makes us feel more confident that the purities which we reported for cells with HDAP were representative of epithelial cells fixed in formaldehyde and stained with the same technique, the Burstone technique, as that used by Stonington et al (14).

We believe that the use of other markers for independent corroboration of the findings with acid phosphatase would be useful. The only other marker which we have employed for prostatic epithelial cells is the lipofuscin granule which is present in a large proportion of human prostatic epithelial cells. There have been numerous reports of immunologically specific acid phosphatase in prostatic cancer, and it is certainly possible that this might be identified in purified cells with a labeled antibody. We have reported several modifications of the procedure for the use of peroxidase labels which have made this approach more sensitive and better suited for the identification of some kinds of cells separated from human tissues in our hands (16–19). Additional markers would be helpful for the characterization of purified prostatic epithelial cells.

Condition and Biochemistry of Separated Prostatic Epithelial Cells

As we have reported (3–5), well over 90% and usually > 95% of the purified prostatic epithelial cells have excluded trypan blue. We have shown that the purified epithelial cells from hamster prostates have a high rate of incorporation of tritiated thymidine in 72-hour cultures (table 1). While we have shown that we can culture purified prostatic epithelial cells from human prostates in short-term cultures, we have not yet tested their rate of incorporation of tritiated thymidine. We have shown that the activities of several enzymes in cultured prostatic epithelial cells change during the course of culture, even in organ culture without dissociation of the tissue into cell suspensions prior to culture. Changes also occur in enzyme activities in purified epithelial cells during the course of culture. Purification of prostatic epithelial cells has provided us with a means of obtaining enzyme which we can be certain had its origin from a specific kind of cell prior to culture. We believe that the purification of cells will provide a very useful means of obtaining isoenzymes from particular kinds of cells. The characterization of isoenzymes from purified cells may prove to be very useful in the more specific definition of serum isoenzymes which are currently being used in most hospitals for diagnostic purposes. As will be reported in detail (20)[3] we have found that acid phosphatase from purified prostatic epithelial cells is neither specifically inhibited by tartrate nor

TABLE 1.—Incorporation of tritiated thymidine by microcultures of purified epithelial cells (100,000 cells/culture) as previously determined (22)

Epithelial cell	Incorporation (mean cpm ± SD)
Prostatic	2705 ± 786
Control*	95 ± 15
Proximal tubule	1342 ± 552
Control	65 ± 2
Urinary bladder	1382 ± 175
Control	150 ± 88

*Control cultures were heated to 56°C for 30 mins prior to addition of tritiated thymidine during terminal 48 hrs of culture. Control cultures which were heat killed were similar to control cultures with a wide variety of poisons including methylazoxymethanol (100 μg/ml).

able to hydrolyze specifically phosphorylcholine. In many hospitals, tartrate inhibitable acid phosphatase is reported as "prostatic acid phosphatase." Seligman et al (21) have stated that their "biochemical experiments" showed "that phosphorylcholine is a very specific substrate for PAP (prostatic acid phosphatase)."

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Culture of Mammalian Prostatic Cells [1]

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SUMMARY

Preliminary experimental data and concepts oriented toward the achievement of certified primary prostatic cell cultures, normal and/or malignant, for mass distribution are presented. Such monolayer cultures would be established as subcultures from the microcarrier, spin-filter (steady-state) system. The suspension culture of primary cells directly derived from the tissue of origin would be propagated at high cell populations for extended periods of time. The ease of complete monitoring on a sequential basis for conformity to specifications is apparent. The feasibility of the approach appears to have been established. Problems relating to the reduction to practice are detailed.

[Cancer Treat Rep 61:161–165, 1977]

The specific objective of our investigations is an evaluation of culture systems and environmental parameters to afford the continued growth of primary epithelial cells representative of those found in the functional parenchyma of the human prostate. We envisaged a system for the direct growth of primary epithelial cells of prostatic origin in long-term suspension culture with maintenance of function, so that this "mother culture" could (*a*) provide adequate, uniform cellular material for genetic, pharmacologic, and biochemical studies; (*b*) provide a source of cells that could be frozen and stored affording a degree of standardization of the cellular characteristics of such primary cells; and (*c*) provide, either from frozen stock or the suspension unit itself, cells on microcarriers that could be used for the rapid initiation of mass monolayer cultures for the screening and assessment of chemotherapeutic, hormonal, and cytotoxic agents that may be of clinical value in cancer of the prostate.

Baulieu et al (1) have suggested that there are several limitations in the reported investigations employing prostatic tissue culture systems. "Thus, to obtain a response to testosterone, a concentration of at least the order of 3.5×10^{-6}M appears necessary, whereas the in vivo plasma concentration of testosterone in the rat is less than 1×10^{-8}M. Moreover, the conditions of *oxygenation*, the *hydrodynamic situation (circulation)* and the absence of plasma binding protein (compensated perhaps by the addition of calf serum?) provoke a turnover of testosterone in the culture differing from that found in vivo. Thus, there is no perfusion of testosterone at the initial concentration, but a constant and rapid decrease of the amount of steroid, since most of it has disappeared after 24 hours, accompanied by an accumulation of metabolites in the system. Such transient states are not encountered in vivo" (1). Our controlled environment system circumvents, to a degree, most of these limitations (2–5).

Prerequisite to establishing the feasibility of this objective is certification that the cultured primary cells are indeed representative of those cells found in the human prostate. For this purpose, functional markers considered for use include the aminopeptidase, acid phosphatase, β-glucuronidase, zinc, citric acid, and acid mucin (sulfated sialomucin) as well as the 5α-reductase levels. At the present stage of our studies, attention has been directed toward a histochemical stain and/or assay of spent culture media

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[2]Roswell Park Memorial Institute, New York State Department of Health, Buffalo.

[3]Our investigations reported in this paper could not have been possible without the cooperation of Angeline Wasielewski. Drs. Avery Sandberg and Rashad Kirdani have collaborated in the steroid studies of the cultured cell. These studies are to be reported in a later publication.

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for specific acid phosphatase (table 1).[4] Morphologic analyses of the cultured cells have been done employing cells cultured on coverslips and/or cryocut sections of cell-covered beads (microcarrier) stained with oil red (Lille Ashburn), Giemsa (May), Trichrome (Masson), and hematoxylin and eosin (Harris): electronmicrograph studies have been carried out also.

It is well recognized that normal and most malignant cells, directly derived from the tissue of origin, will not grow in suspension culture. They can, however, be frequently cultured as monolayers on various surfaces. Efforts have been made to devise various surface-type fermentor configurations to take advantage of this characteristic. The most promising appears to be the observation that primary mammalian cells could grow on the surface of beads of DEAE-Sephadex A-50 (8), and that such beads could be kept in suspension by gentle stirring. Thus, primary suspension-microcarrier cultures came into being.

It is apparent that a controlled environmental configuration coupled to the primary microcarrier suspension system for the in vitro culture of mammalian cells appears desirable for one interested in studies of the in vitro morphology and function of the mammalian cells, as well as the precise role of potential growth regulators on cell metabolism.

Conventional monolayer culture flask configurations deal with a batch media change schematic permissible because one is dealing with dilute cell populations maintained at low levels. This in turn signifies low levels of total substrate consumption, glucose, etc, as well as lactate elaboration. pH shifts, while significant, occur gradually over a 2- or 3-day period between media changes. Increasing the cell population fivefold or tenfold to achieve the desired "tissue mass effects" also results in minimal use of serum as coupled with an efficient operational attitude. This, in turn, results in an intensive acceleration of substrate consumption and lactate production. To counter this and avoid the traumatic effects of excessive pH changes, one must make media changes at ever-increasing frequencies until at last one reaches the steady-state configuration. An example of experimental data supporting this view is detailed by Noyes (9) and Gailani et al (10,11).

Long-term steady-state organ cultures of human prostatic tissues have been studied in the thin-film rocker system (9). There have been a number of reports of the conventional organ culture of human benign hyperplastic prostatic tissue with maintenance of good histology for brief periods up to 9 days. Noyes (9) reported, on the other hand, the maintenance of normal human prostatic tissue in organ culture for prolonged periods, 15–30 days, by employing the steady-state system. Thus, long-term organ cultures of normal human prostatic tissue with demonstration of functional and morphologic maintenance of the glandular epithelium was described. The carcinogen, 3-methylcholanthrene, at a level of 4 μg/ml in the culture induced morphologic changes that were not observed in untreated cultures. These changes were suggestive of a neoplastic potential.

Of great importance are the reports of Gailani et al (10,11) on their experience with human bone marrow organ culture, performed under steady-state conditions using the thin-film, rocker unit. Such cultures have been maintained for periods of at least 40 days with significant evidence of continued function. They showed that 2 to 4 times as much IgG was elaborated from organ cultures maintained at pH 7.4 than at pH 7.1. The preset oscillation of pH between the above two pH limits resulted in "tracking" as demonstrated by the IgG production levels. Moreover, it was conclusively demonstrated that while 0.5–2 μg/day of IgG was produced by stationary, conventional-type, bone marrow cultures,

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[4]Culture evaluations were carried out using (*a*) a monolayer culture system as contained in a specially designed "Bell Jar," and (*b*) monolayer steady-state (S-S) thin-film units (2–4) and/or direct primary cultures on microcarriers in an S-S suspension culture system. All monolayer culture surfaces consisted of calf tail collagen. The basic culture medium was McCoy's 5-A adjusted to 100 or 400 mg/100 ml of glucose. The media were supplemented with 10% native serum of various species, 1 μg/ml of hydrocortisone, 5 μg/ml of insulin, and as indicated 0.1 μg/ml of testosterone and/or 0.1 mg of zinc/ml. The precise adjustment of pH via the tonometer has been described. The antibiotic, gentamicin, was used as the only antibacterial agent in the culture system. For S-S evaluation, we used the thin-film, monolayer, rocker system wherein the media fed to the culture could be programmed in ranges from 1- to 6-hour intervals while maintaining a constant volume, pH, and gas atmosphere (2–6).

The acid phosphatase reactions encompassed the following (7):

(1) a-naphthyl phosphate $\xrightarrow{\text{enzyme}}$ a-naphthol + H_3PO_3

(2) a-naphthyl + Fast Red Salt B (FRSB)
a-naphthol FRSB (absolute at 588 nm)
$\xrightarrow{\text{0.1 N NaOH}}$ colored complex

Our acid phosphatase assay is a modification of the Babson-Phillip procedure (7), where 100 μl of specimen is incubated for exactly 15 minutes in an a-naphthyl phosphate-citrate buffer solution (3 nM; 0.2 M, pH 5.6, respectively) at 37°C. The hydrolyzed a-naphthol is measured by the addition of 1 ml of FRSB solution (20 mg/100 ml, 0.1 N HCl) and the reaction is arrested and the color developed with 5 ml of 0.1 N NaOH. The specimen is read at 588 nm against its respective blank, which is treated in the same way as the test with the exception of adding the 100 μl of specimen after all other reagents.

The activity is calculated by comparing the ratio of the color produced to an internal (instrumental) primary standard, and the results are reported in IU/liter (or milliunits/ml) 37°C.

TABLE 1.—De novo synthesis acid phosphatase by human prostatic cell monolayers as cultured under steady-state conditions via the thin-film rocker unit: tissue of origin = human prostate (SS-99)

Elapsed time (hrs)	Spent culture media		Acid phosphatase*		Control media
	cc/tray/day	pH	MU/cc	MU, net	
691	60	7.32	0.9	12.0	
715	58	7.31	0.9	11.6	
739	53	7.32	0.9	10.6	
763	38	7.44	0.8	3.8	
787	60	7.32	1.0	18.0	
811	56	7.34	1.2	28.0	0.6
835	66	7.33	0.8	6.2	0.9
859	68				0.8
885	58	7.34	1.0	17.4	0.5
907	57	7.34	1.0	17.1	
			Analysis		
Mean	57.4	7.34	0.94	13.86†	0.70
Range	38–68	7.31–7.44	0.8–1.2	3.8–28.0	0.5–0.9
Span	30.0	0.13	0.4	24.2	0.4
SE	2.58	0.01	0.04	2.41	0.09

*Acid phosphatase level/flask/day = $\frac{13.86}{5}$ = 2.77 milliunits (MU).

†Milliunits: (vol spent media) – (milliunits control media) · (vol spent media) = net.

paired specimens under steady-state configurations produced 17–20 μg/day, a 10–18-fold increase (10,11).

Culture parameter investigations revealed that a mince-type tissue explant of the original prostatic tissue—human or baboon—rapidly yielded cells that attached to the Sephadex beads. This occurred in stirred suspension and/or as the tissue was layered over a fixed bead bed. Similar cell attachment and growth was realized when trypsin-dissociated cells engaged the bead surface. The nature of the ensuing growth pattern strongly suggested routine use of the tissue mince inoculum.

Optimal growth conditions employed were pH 7.35, pCO_2 2% (baboon) and 5% (human), osmolarity 310–315 milliosmols, and media feed rates of 1%–10% of the culture volume per hour and/or as determined by culture pH signals. The culture media consisted of McCoy's 5-A supplemented with native horse serum, testosterone, zinc, insulin, and hydrocortisone.

Under the described culture conditions employed with a 500-ml microcarrier, spin-filter, suspension system (MSF), excellent direct cell attachment to the beads was realized with sustained primary growth of prostatic cells (fig 1). Morphologic analysis was made by direct section and staining of the cell covered beads and/or by subculture clones as established by plating diluted aliquots from the MSF fermentor. Following a 7-day incubation period confluent cellular monolayers of uniform epithelioid configurations were produced. Supernatant fluids from the MSF fermentor yielded high levels of specific acid phosphatase activity, as well as 5α-reductase activity of cellular origin.

It appears that the feasibility of providing uniform primary prostatic cells, in mass, for distribution to other laboratories (interested in, for example, the chemotherapy of prostatic cancer) has been established. Before being fully functional as a "mother" culture system, problems relating to the characterization of cells obtained from various types of human cancer will require careful study. Cultures derived from the normal baboon prostate may serve as a useful control. Additionally, much further work on functional certification of such cells, prior to routine use, appears mandatory. Finally, we need to search for and evaluate other nutritional or hormone supplements and culture parameters.

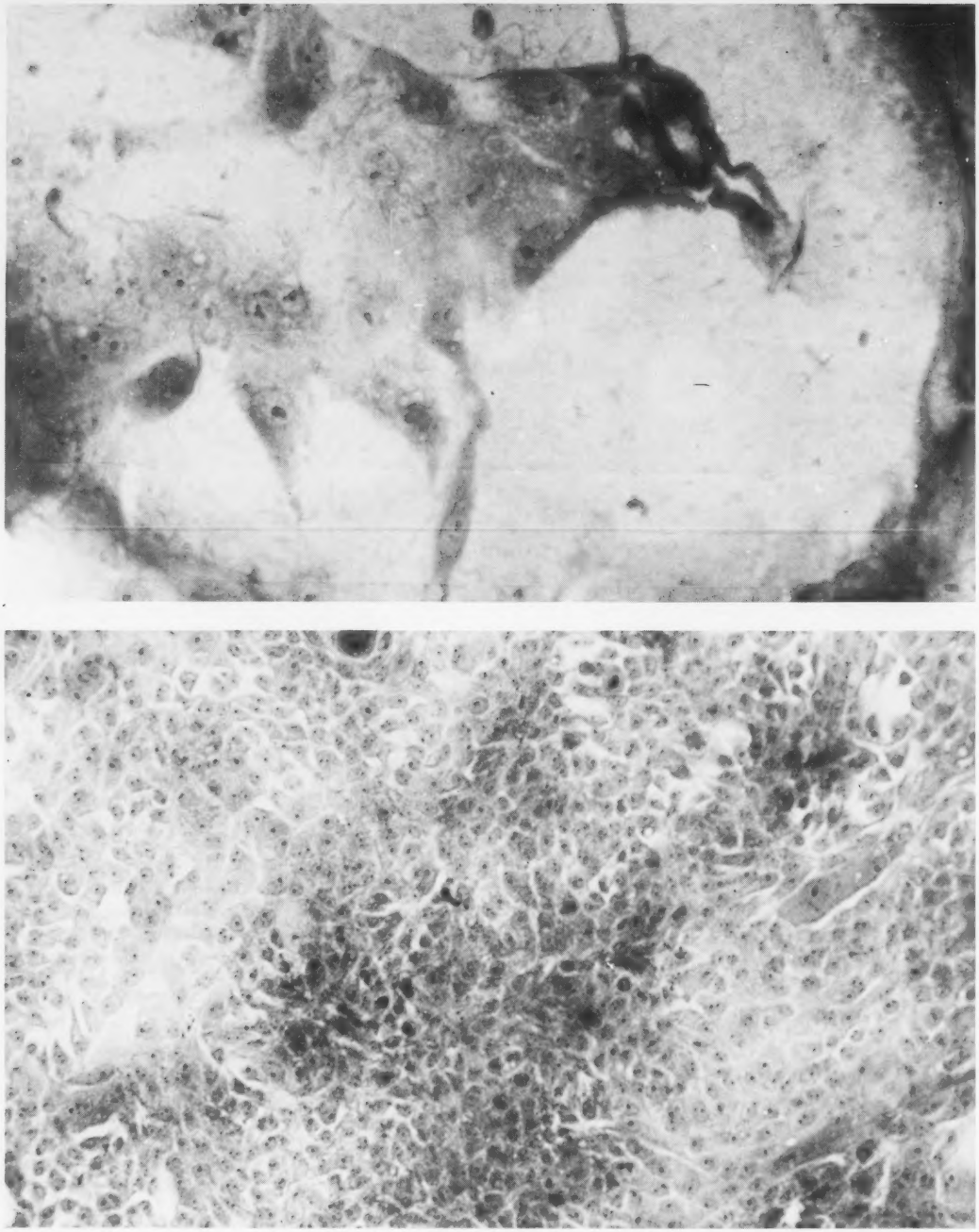

FIGURE 1.—7-day monolayer cultures of normal prostate (baboon) derived from microcarrier spin-filter culture (235.5 hrs). Top view shows cells on collagen bead (× 900). Bottom view shows hematoxylin and eosin stained monolayer (× 100).

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Characterization of Prostatic Carcinoma Among Blacks: A Continuation Report [1]

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SUMMARY

A clinical and pathologic comparison of carcinoma of the prostate in a high-risk US (Washington, DC) black population and in a low-risk Nigerian (Ibadan) black population is presented. Fifty-two percent of American patients were in clinical stages I and II, whereas only 10% of Nigerian patients were in the same stages. Testosterone and estradiol blood levels were significantly lower ($P < 0.05$) in patients with carcinoma of the prostate from Ibadan compared with American black prostate cancer patients from Washington, DC. Age-standardized incidence rates (world standard) for 1000 autopsies for micro (incidental)-carcinoma were approximately equal in American and African black men. The incidence rate of invasive carcinoma was, however, even after adjustment for age, higher in American black men than in African men.

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The age-adjusted death rate for carcinoma of the prostate among 24 countries surveyed throughout the world was highest in US non-white men (22.73/100,000) for the period 1966–67. In these 24 populations, US non-white men ranked first in order and US white men ranked 15th in order (12.61/100,000) (1).

Calculations based upon 1973 data show the probability at birth of developing cancer of the prostate in the US was 6.4/100,000 for black men and 5.1/100,000 for white men. The probability at birth of dying of carcinoma of the prostate in the US was 2.4/100,000 for black men and 1.8/100,000 for white men (2).

In Washington, DC, the age-standardized incidence rate (world standard) for black men was 59.8/100,000, while in Ibadan, Nigeria, the corresponding incidence rate was 9.7/100,000 (3).

Whether the high rate of invasive prostatic carcinoma in US black men is preceded by a similarly high rate of incidental carcinoma of the prostate is not known. Only a few studies have been done on carcinoma of the prostate in Africa. In a limited study of prostate glands from 96 African patients (89 of them from Uganda), there was definitive evidence of incidental carcinoma in ten patients and possible evidence in an additional eight patients (4). In contrast, the incidence rate for invasive carcinoma in Ugandan men was 4.4/100,000 (5).

MATERIALS AND METHODS

Patients

All new, untreated patients with carcinoma of the prostate gland were admitted to the study. Blood samples for hormone and phosphatase analyses were drawn from all male patients after admission to the Urologic Services of Howard University and DC General Hospitals in Washington, DC, and University Hospital, Ibadan. The blood samples were collected under constant fasting pre-medication conditions at 8:00 AM and before examination of the prostate gland. Plasma and serum samples were frozen and stored until a histologic diagnosis of carcinoma of the prostate was established. Total alkaline (6) and acid and prostatic acid (7,8) phosphatase activities were determined from the serum samples. The plasma sample was analyzed for testosterone, estrone, estradiol (9,10), and prolactin.

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Blood samples taken from patients without carcinoma of the prostate were discarded. Patients with histologically confirmed prostatic carcinoma were subjected to a detailed clinical evaluation and staged according to the Veterans' Administration Cooperative Urological Research Group system (11).

Epidemiology

All patients with histologically confirmed carcinoma of the prostate and all control patients were interviewed using pre-tested epidemiologic questionnaire forms (12). Hospital controls were chosen for this study.

Control patients, age-matched with prostatic carcinoma patients, free of clinically demonstrable neoplastic, urologic, and endocrine conditions, had not received hormonal therapy within the past 6 months and had not received blood transfusions within 3 days preceding the collection of blood samples for hormonal and enzyme analyses.

Morbid Anatomy

The whole prostate gland was collected from consecutive necropsy cases of black men in Washington, DC, and Ibadan. Whole glands with attached seminal vesicles were accessioned, entered into a blind study, weighed, step-sectioned at 4-mm intervals, processed, mounted on large slides, and stained with hematoxylin and eosin (13). In addition to the prostate glands, tissue blocks of liver, adrenal gland, testis, pituitary gland, and breast were collected from each case, processed, mounted, and stained with hematoxylin and eosin. Each case was analyzed for specific morphologic changes according to the protocol.

From patients with the clinical diagnosis of carcinoma undergoing prostatic surgery, sections from all tissue blocks were studied. The histologic grade of the tumor and other morphologic parameters were correlated with the clinical staging, hormonal studies, and epidemiologic data. All tissue specimens for this study were evaluated in one laboratory.

RESULTS AND DISCUSSION

Sixty-five Nigerian patients with histologically confirmed carcinoma of the prostate, 33 Nigerian control patients, 243 Nigerian necropsy cases, 132 US patients with prostatic carcinoma, 99 US control patients, and 249 necropsy cases were entered into the study through March 31, 1976. Tissue sections from either transurethral resection, perineal needle biopsy, or both were studied from 65 Nigerian and 132 US cancer patients. Complete clinical data were available for all 65 Nigerian prostatic cancer patients, nine of the 33 Nigerian control patients, 114 of the 132 US cancer patients, and 87 of the 99 US control patients; complete hormone analyses were available for 31 of 65 Nigerian cancer patients, five of 33 Nigerian control patients, 94 of 132 US cancer patients, and 76 of 99 US control patients. (The number of Nigerian control patients available for analyses is small and interpretations based upon this limited material should be made with caution.)

The distribution by clinical stage of patients with carcinoma of the prostate (table 1) shows that 52% of US and 10% of Nigerian patients are in stages I and II (low stage) while 48% of US and 90% of Nigerian patients are in stages III and IV (high stage). There was considerable difference in the two surveyed US hospitals: DC General and Howard University. Thirty-nine percent of the prostatic cancer patients at the DC General Hospital were in low stage (stages I and II) and 60% of the prostatic cancer patients at Howard University Hospital were in low stage when first seen. This difference suggests that the stage of the disease in the patient on first contact with the physician may be a function of socio-economic conditions. The interstage age variation is not significant. A comparison with a racially unselected series from Roswell Park Memorial Institute (Buffalo, NY) (14) shows that whereas a large proportion of patients from Howard University and DC General Hospitals were in low stages, the percentage of patients in stages I and II was relatively small at Roswell Park Memorial Institute (fig 1). The low relative frequency of patients in stages I and II in Ibadan is most likely due to the fact that those patients are at an advanced stage when first diagnosed. Most cases of prostate cancer at Howard University and DC General Hospitals are newly diagnosed cases of the disease and are probably therefore in the low stages. Roswell Park Memorial Institute is a large referral center and that explains the predominance of prostate cancer patients in high stages.

Mean plasma testosterone, estradiol, and estrone levels (table 2) were significantly lower ($P < 0.05$) in Nigerian than in US prostatic cancer patients. Nigerian cancer patients showed a significant difference in mean testosterone levels by clinical stage at a level < 0.05. Nigerian control patients, although few in number, showed a higher mean plasma testosterone level than Nigerian prostatic cancer patients. No significant difference was observed between mean plasma testosterone levels and clinical stage in US cancer patients at the 0.05 level. There was no significant difference in mean plasma estradiol levels by clinical stage in either US or Nigerian cancer

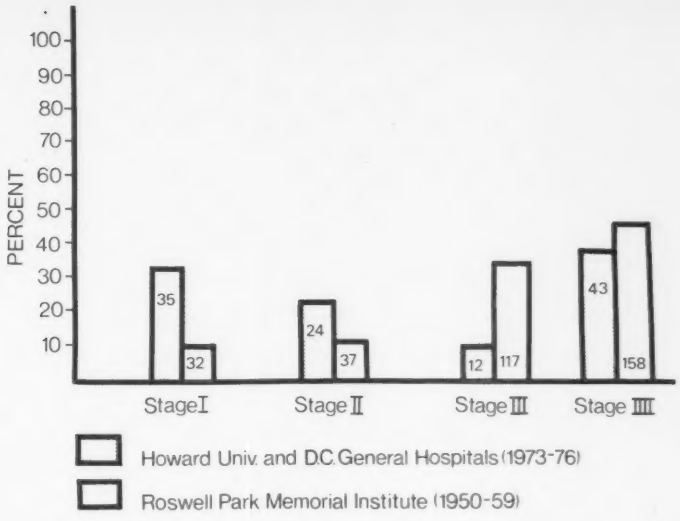

FIGURE 1.—Comparison of staging of patients with carcinoma of the prostate in Howard University and DC General Hospitals (Washington, DC), and Roswell Park Memorial Institute (Buffalo, NY).

patients. The mean plasma estrone levels significantly differed by clinical stage at a level of 0.05 in US cancer patients. The mean levels increased with increased stage. A significant difference in mean estrone levels was not observed in Nigerian cancer patients. Mean plasma prolactin levels were not significantly different between prostatic cancer patients and controls nor between cancer patients in the two population groups.

Tissue was examined from all patients with carcinoma of the prostate. The age distribution of these patients (fig 2) showed that the peak age for Nigerians was in the 55–64-year group which is a decade earlier than for US blacks in Washington, DC.

In comparing American and Nigerian black patients in the two age groups with the highest number of cases (age groups 55–64 and 65–74 years old), there was a marked predominance of grade I carcinoma over grades II and III in US blacks (fig 3). However, this was not noted in Nigerian men. A comparison of the mean ages of black patients with grades I–III carcinoma revealed that the mean ages of patients for all grades were higher in US black men than in Nigerian men. For all grades the age difference between US and Nigerian black patients was significant ($P < 0.05$). The difference in the age distribution of black men with carcinoma of the prostate from Ibadan and Washington, DC, may reflect the difference in age structure of the two populations. The preponderance of cases with lower grades in the US patients may be related to the earlier diagnosis of the disease in this country.

Epidemiologic data from 99 US patients and 95 matching controls indicated that a statistically significant association existed between the following variables and prostatic carcinoma: (*a*) younger estimated real age; (*b*) less use of aphrodisiacs; and (*c*) history of bronchial asthma. An association close to the $P < 0.05$ level was found between prostatic carcinoma and the following variables: (*a*) more perspiration during work; (*b*) lower number of different sexual partners per month during the period 10–19 years before diagnosis of illness; (*c*) family history of cancer; and (*d*) higher consumption of hard liquor ≥ 10 years before diagnosis of prostate cancer.

Twenty-five Nigerian patients and controls were matched and their epidemiologic data were reviewed. The number of responses to various questionnaire items, after exclusion of the "no answer," were too few to obtain a meaningful analysis.

Two hundred and forty-three necropsy cases from Ibadan and 249 necropsy cases from Washington, DC, were analyzed (table 3). In 5.8% of the autopsies from Nigeria and in 19.3% of the autopsies from Washington, DC, there was carcinoma found in the prostate gland. The median age of cases with carcinoma of the prostate was 20 years higher in Washington, DC, than in Ibadan. The crude and

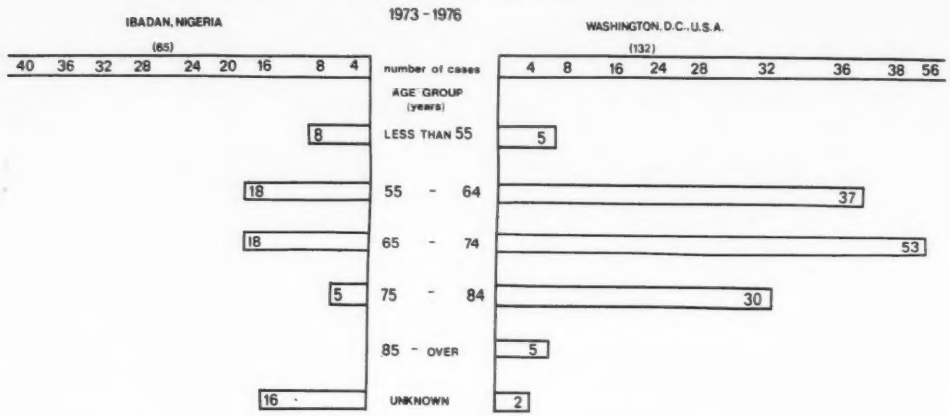

FIGURE 2.—Age distribution of prostatic carcinoma in surgical material (1973–76).

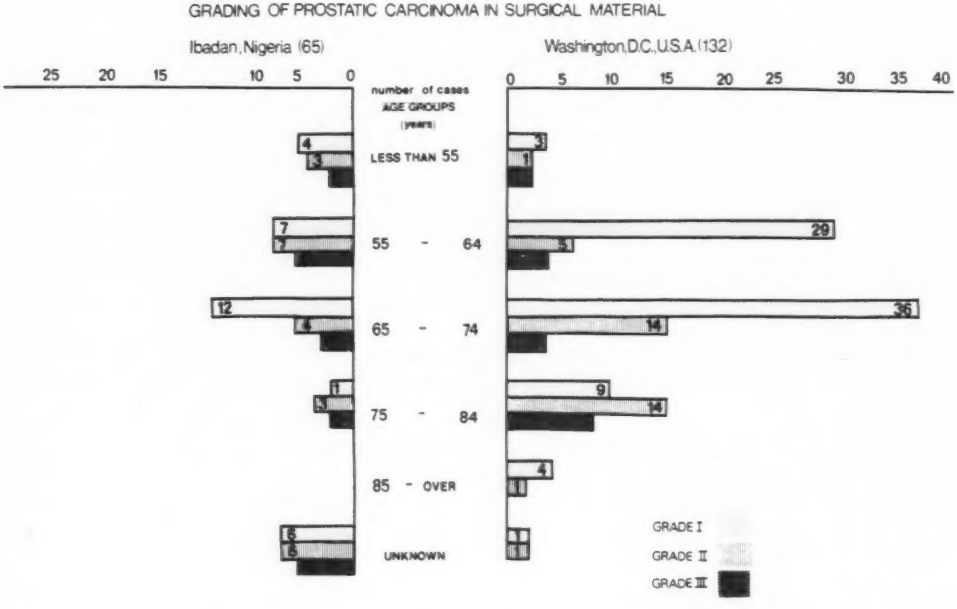

FIGURE 3.—Grading of prostatic carcinoma in surgical material (1973–76).

TABLE 1.—Comparison of staging of black patients with carcinoma of the prostate in Washington, DC, and Ibadan

Disease stage	Washington, DC		Ibadan	
	No.	%	No.	%
I	35	30.7	3	5
II	24	21.0	3	5
III	12	10.5	15	24
IV	43	37.8	41	66
Total	114	100.00	62	100

TABLE 2.—Mean hormone levels in prostate cancer and control patients in Washington, DC, and Ibadan

Patients	Hormone levels ± SD		
	Testosterone (ng/ml)	Estradiol (pg/ml)	Estrone (pg/ml)
US			
Cancer	4.80 ± 2.20	32.86 ± 10.76	44.0 ± 12.0
Control	4.36 ± 1.58	32.25 ± 10.62	38.45 ± 10.91
Nigeria			
Cancer	2.15 ± 1.20	26.50 ± 2.76	35.20 ± 10.0
Control	3.78 ± 0.74	26.50 ± 2.80	39.0 ± 10.28

TABLE 3.—Incidence of prostatic carcinoma in necropsy cases

	Ibadan	Washington, DC
Total No. of cases	243	249
With carcinoma	14	48
With micro-carcinoma	8	18
With invasive carcinoma	6	30
Age of cases with carcinoma (yrs)		
Mean	48.75	71.1
Median	46.0	69.5

TABLE 4.—Crude and age-adjusted incidence rates for carcinoma of the prostate (No. per 1000 necropsies)

Carcinoma	Ibadan		Washington, DC	
	Crude	Adjusted*	Crude	Adjusted*
Micro	32.9	25.5	72.3	23.5
Invasive	24.7	18.5	120.5	27.9
Total	57.6	44.0	192.8	51.4

*Adjusted against the world standard population.

age-standardized incidence rates for carcinoma of the prostate per 1000 autopsies are shown in table 4. The crude rate for this type of malignancy was three times higher for blacks in Washington, DC, than for Nigerians in Ibadan. After adjustment for age, however, the crude rates approximated each other. The crude rate for micro (incidental)-carcinoma was twice as high in blacks from Washington, DC, than in blacks from Ibadan. Age adjustment showed an almost equal rate of this type of cancer in the two population groups. The rate was, however, slightly higher in Nigerians. The rate for invasive prostatic carcinoma was five times higher in autopsies from Washington, DC, than in necropsy material from Ibadan. After age adjustment the incidence rate per 1000 autopsies was still 9.4/1000 cases higher in the necropsy material from Washington than in the autopsies from Ibadan. These findings suggest that factors which stimulate the proliferation of neoplastic cells in the prostate in the western world are less apparent in the African continent.

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Studies in the Epidemiology of Prostatic Cancer: Expanded Sampling [1]

I. D. Rotkin [2,*]

SUMMARY

From comparisons of prostatic cancer patients with matched control patients for selected risk variables, patients are characterized by three main trends: (*a*) delayed sexual drive and development, (*b*) early repression of sexuality, and (*c*) premature cessation of sexuality. Excessive numbers of patients reported occupational exposure to fertilizers and auto exhaust fumes. Diets of the patients were higher in animal fats. No differences were found between both groups for frequencies of multiple marriages or sex partners, nor for stressful effects from selected events early or late in life. Trends for circumcision and other variables are presented. The data suggest that early differences are hormonally conditioned, support a provisional endogenous rationale for initiation of prostatic cancer, and oppose a hypothesis favoring transmissible oncogenic agents. If results continue to hold up with increased sampling, limitation upon sexual activity at any time of life may increase risk.

[Cancer Treat Rep 61:173–180, 1977]

A first appraisal of grouped epidemiologic data from samplings of 86 prostate cancer patients and 74 controls, recently presented in New York (1), was useful for the development of emerging trends. Some of these trends were resolved, which provided a rationale for increased risk of prostate cancer, and could be fitted to the classic continuum of carcinogenesis. For this report, 111 matched pairs of patients and controls are evaluated with regard to those trends and differences which persist, and those which so far do not appear to characterize prostatic cancer patients as a separate population when compared to controls.

Trends from the first report supported an endogenous effect upon the initiation of prostate cancer. Since patients developed secondary sexual characteristics later than the general male population, the influence upon transformation of prostate epithelium provisionally was regarded as hormonal. Patients could be characterized by a delayed onset, an earlier peak, and a premature falloff of coital sexuality later in life, with evidence for sexual repression rather than a diminished libido both early and late in life. The data delineated late adolescence and the early twenties as a specific time of life when patients differed most from controls. Two occupational exposures were identified in relation to risk: fertilizers and auto exhaust fumes. Results from the first study could not be interpreted to support the effect of a venereally transmissible oncogenic agent, and it was concluded that if there is an exogenous contribution to risk, it would come from occupational or other nonsexual origins. In general, most major trends from the first report are supported by the current expanded sampling.

PROCEDURES

The design is a matched-pair multivariate exploration for factors which increase risk of prostate cancer, working from retrospective information collected with interview-administered questionnaires covering 20 domains of defining variables. The objective is to eliminate variables with no demonstrable effect upon the origin and development of prostate cancer, and to identify those possibly contributing to risk variance. The methodology is complex but is manageable with procedures developed for studies in cervix cancer (2).

Samplings of patients are from two of the three

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TABLE 1.—Percent distributions by age and race: prostate cancer patients and controls (111 pairs)

Age class	Nonwhite		White	
	% of patients (total, 38 patients)	% of controls (total, 42 controls)	% of patients (total, 72 patients)	% of controls (total, 69 controls)
< 55	0.0	2.4	0.0	1.4
55–64	28.9	31.0	20.8	23.2
65–74	39.5	40.5	47.2	39.1
75–84	28.9	26.2	29.2	33.3
> 85	2.6	0.0	2.8	2.9
	99.9	100.1	100.0	99.9

TABLE 2.—Comparison of mean ages of samples by institution and race (111 pairs)

Race	Kaiser Foundation Hospital		University of Illinois/West Side VA Hospital	
	Patients	Controls	Patients	Controls
White	71.8	71.1	66.9	67.0
Nonwhite	69.5	68.4	69.0	68.4

TABLE 3.—Comparison distribution by means of age classes: prostatic cancer patients and controls, combined hospitals and races (111 pairs)

Age classes	Mean ages	
	Patients	Controls
> 55	—	48.5
55–59	57.7	57.9
60–64	62.6	62.7
65–69	66.4	66.4
70–74	72.0	71.6
75–79	77.1	77.1
80–84	80.5	81.6
85–89	86.0	88.0

participating institutions, the Kaiser-Permanente Medical Center, Los Angeles, Calif, and the University of Illinois College of Medicine, Chicago, with patients and controls for the latter taken from the associated West Side Veterans' Administration Hospital. Additional sampling is now under way at the third institution, the Northwestern University Medical School and Hospitals, Chicago, Ill.

Prostate cancer patients are identified from tumor registries and rosters at each institution. Outpatient charts and hospital appointment lists are combed daily for controls, and are identified as they correspond to prostate cancer patients by age and race. Each matching control is qualified by digital rectal examination for admission in the study. Exclusion criteria for controls are history of any other neoplasm, failure to match a sampled patient, prostatectomy for any reason, history of any prostatic or urologic problem except prostatitis, inability or unwillingness to participate in the research, and the presence of any clinical degree of benign hypertrophy or other suspicious pathology. Information is recorded in a separate data record which is structured to preserve subject anonymity and confidentiality. The purpose of the study is fully disclosed to the patients and controls.

Results are presented here as bar diagrams from grouped and distributionally matched data in a form suitable for appraisal of excesses for each variable as they developed from comparisons of patients and controls. Even when such excesses do not reach the confidence levels customarily selected as significant, they can provisionally be interpreted in relation to increasing risk when consistent with other associated variables trending in the same direction.

RESULTS

Distributions of patients and controls by age and race are parallel (table 1), with some minimal variation arising artificially from the separation of some pairs into adjacent age classes. However, means for patients and controls at both institutions are alike (table 2), and comparison of means by individual age classes demonstrates a very close equivalence (table 3).

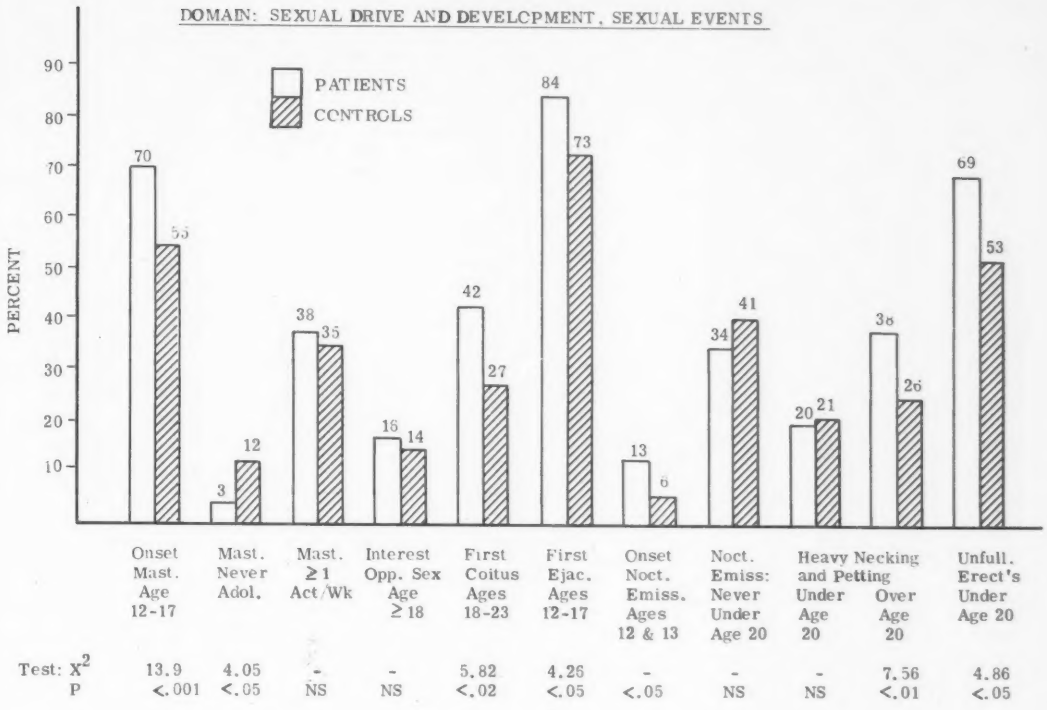

FIGURE 1.—Variables related to sexual drive and development.

Demographic observations in the original report (1) for multiple marriages and military service have held up with only slight but nonsignificant excesses of patients who were married more than once and who served > 3 years in the Armed Forces. However, the originally reported excesses of Catholic patients and deficits of Jewish patients disappeared in this expanded sampling.

A series of comparisons related to sexual drive and development is shown in figures 1 and 2. In general, trends and relationships which developed earlier have persisted, with significant excesses of patients who began masturbatory and coital activities later in life than controls. More patients than controls also experienced a first ejaculation later than controls, although more controls had not begun to masturbate during adolescence. From the delayed interest in females by more patients than controls, a deficit in libido might be attributed to patients, but this effect also can be interpreted as a repression of sexuality. This is suggested by trends showing more patients with sustained masturbatory activity and with onset of involuntary nocturnal emissions at the beginning of adolescence, a result that is strengthened by the excess of controls who had never experienced the well-known stress of unfulfilled erections upon the genitalia and reproductive organs during adolescence. This effect not only is physiologic but is also an indicator of sexual drive. However, from the large percentage of patients with unfulfilled adolescent erections, an interpretation again is possible favoring early repression of sexuality rather than lack of libido for patients. Activities covering sexual seduction and foreplay referred to as *necking* and *petting* also provide insight into sexual drive and reveal larger frequencies of patients who delayed these activities past adolescence, confirming again delayed onset of sexuality for patients.

All findings from the review of current results in figures 1 and 2 parallel those in the earlier report. From figure 2a, there is no difference between

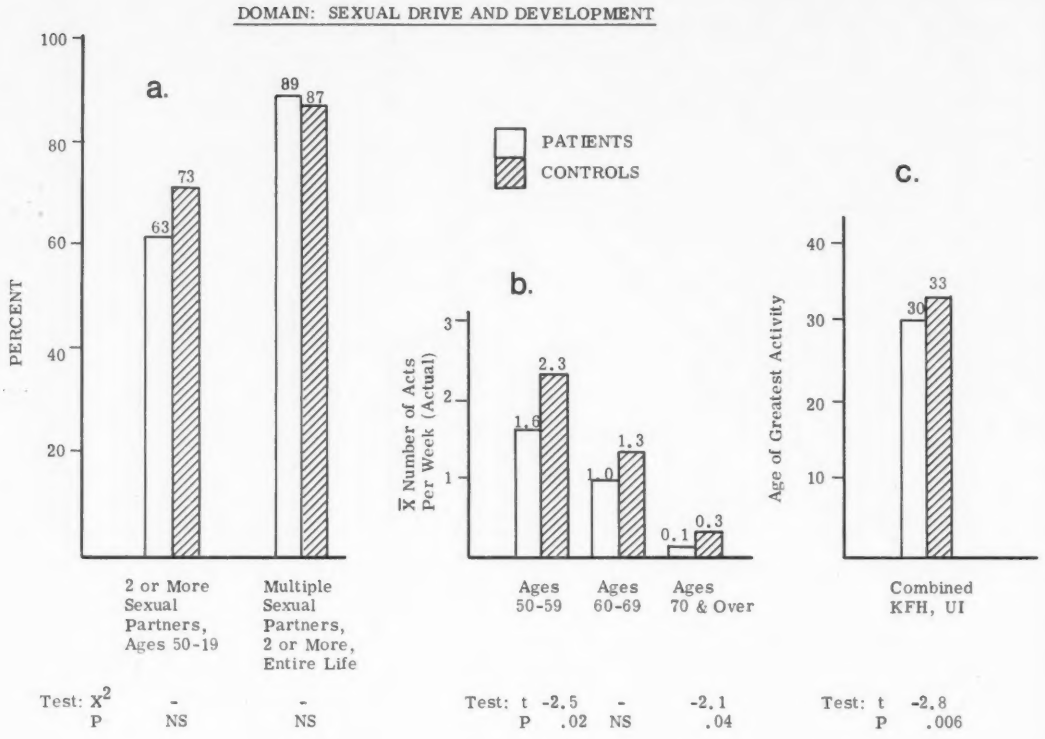

FIGURE 2.—Events related to sexual drive and development: (*a*) multiple sexual partners, (*b*) acts per wk in later decades, (*c*) age of peak activity.

frequencies of patients and controls who experienced multiple sexual partners at any time in life, a finding that opposes the notion of a transmissible oncogenic agent, as also did the similarities of multiple-marriage frequencies for both groups.

A consistent deficit of sexual frequencies for patients covering all age groups after 50 years of age is shown in figure 2b, indicating a falloff of activity early in life for patients. There was the further suggestion that patients generally might be characterized by lower levels of activity early in life than the general male population ($P = 0.07$, data not shown). However, differences between patients and controls were not substantial, and this comparison serves only to emphasize the profile of the prostate cancer patient who has repressed or delayed sexuality past normal onset. Patients also arrived at an earlier peak of sexual activities (fig 2c) by about 3 years. The total impression is one of a short activity span for patients, beginning late in life, peaking earlier, and tapering off or terminating at an early age. This diminution of sexual activity coincides with the age period when prostate cancers begin to appear in the population.

There is reason to believe from developing data that the endogenous influence upon initiation of the carcinoma during adolescence is hormonal. Figure 3 demonstrates a retarded development of several secondary sexual characteristics by more patients than controls, the age spans shown being late for onset of these variables. While not impressively large, differences for change of voice pitch, development of pubic, axillary, and chest hair, and enlargement of pubertal genitalia are all in the same direction, as were the earlier findings. It is possible that the period of life from 15 to 20 years of age represents a specific time when prostate cancer patients differ most substantially from the rest of

COMPARISONS, PROSTATE CANCER PATIENTS AND CONTROLS

DOMAIN: HORMONAL, ONSET SECONDARY SEXUAL CHARACTERISTICS

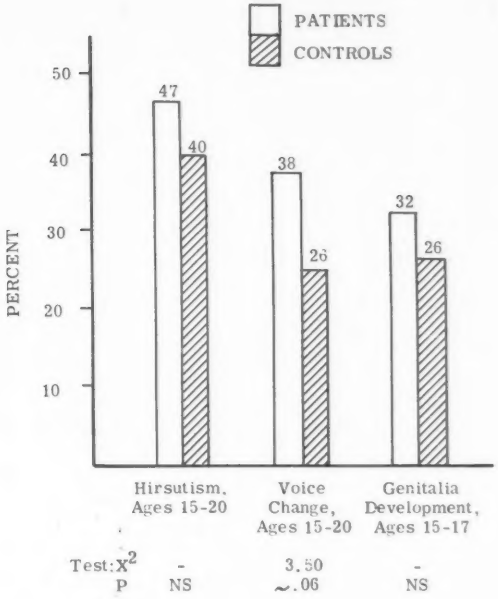

FIGURE 3.—Hormonal variables, onset of secondary sexual characteristics.

the male population, and that the issue here is not retardation of development but rather specificity of scheduling, especially since excesses of controls developed these secondary sexual characteristics before and after this time of life.

Certain other characteristics, perhaps associated with hormonal maturity did not appear to follow this trend. Similar frequencies of patients and controls began to shave during early adolescence, and an excess of controls began to shave later in life contrary to earlier findings, but shaving may not be entirely relevant to hormonal activity since it also is a behavioral practice that is subject to conditioning as a socialization process. There were also no perceptible differences at this stage of the research in physical growth rates between patients and controls for ages 12–17.

An exploratory group of variables is included in the questionnaire to search for the effect of stressful periods in life upon risk, two of which are shown in figure 4a. An excess of controls is shown to have suffered from broken homes early in life; and although more patients than controls were found with a history of a severe depression immediately preceding detection of the cancer, the difference is small, and relatively few in either group experienced such a depression. From these variables no readily apparent contribution to risk from psychologic stress can be perceived. Again, results parallel those from the earlier report.

More patients than controls were exposed to fertilizers or to auto exhaust fumes as part of a consistent occupational pattern (fig 4b). Both effects are interesting since the nitrogen content in fertilizers can undergo biosynthesis into nitrosamines, and the carcinogens in auto exhaust fumes are known; but these interim results alone do not constitute justification for the identification of fertilizers and auto exhaust fumes as extraordinary hazards related to prostate cancer. Still, the current data support the earlier findings, and it will be necessary to await completion of the study before decisions can be reached regarding the adoption of these results as hypotheses for further testing. Should they be confirmed, there might be reason to believe that exogenous influences which are nonsex-

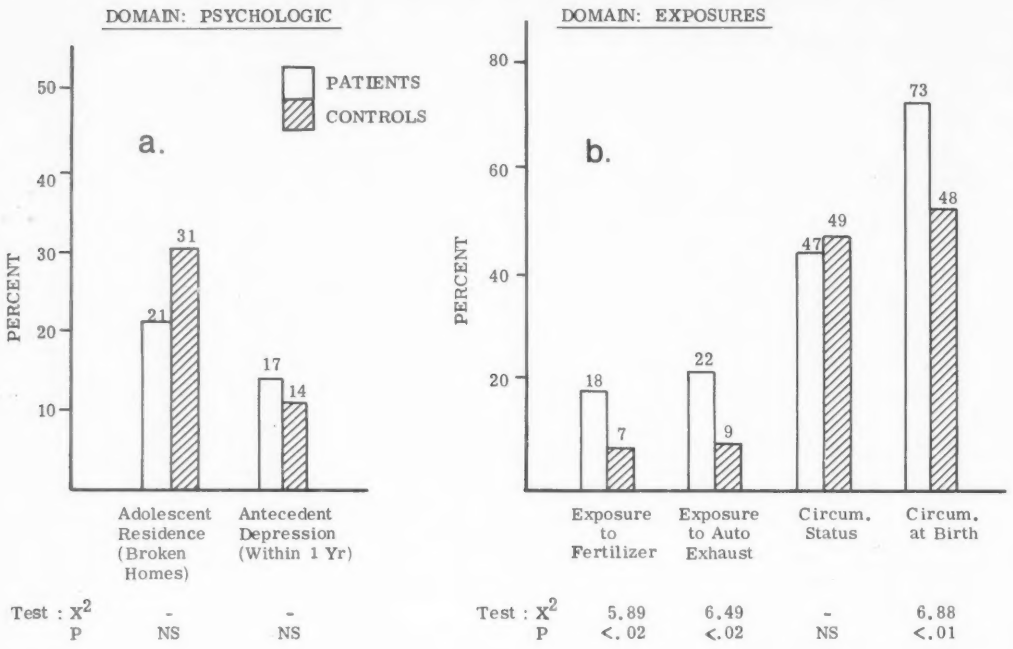

FIGURE 4.—(*a*) Selected psychologic variables related to stress early and late in life, (*b*) occupational exposures and circumcision.

ual may increase risk independently or in combination with genetic and other endogenous forces.

Frequencies of circumcision by status remained almost identical for patients and controls, but in the current review of data an excess of patients had been circumcised at birth, the meaning of which is difficult to interpret at this stage since it is in an unexpected direction.

Reported for the first time from this study, certain foods were selected to estimate differences in the ingestion of fats between patients and controls. Wynder et al (3) have expressed the impression that lipids may have an effect upon prostate cancer risk. From results in figure 5, it is difficult to escape the feeling that some components which are heavy in lipids and cholesterol are represented in greater quantities for patients. Independent results from the Kaiser-Permanente and the University of Illinois-Veterans' Administration samples are shown in a search for consistency. More fatty meats, eggs, cheeses, and creams were consumed by patients at both institutions, with the exception of meats by the Kaiser sample. Significantly more eggs were consumed by patients at both institutions.

DISCUSSION AND CONCLUSIONS

In this review of 111 pairs of patients and controls, the findings from the preliminary report were largely upheld. Patients with prostate cancer still appear to be distinguished from the general population of men by three main currents: (*a*) delayed sexual drive and development, (*b*) repression of sexuality rather than a lack of libido at the time in life when sexual activities normally would begin, and (*c*) a falloff of sexuality early in later life. The specific age period in early life when differences between patients and controls were most dramatic was during late adolescence and perhaps the early twenties, at which time it is possible that the prostate is most vulnerable to cellular transformation and the initiation of the carcinoma. If these observations continue to hold up as they have so far, limitation upon sexual activity during adolescence and cessation of sexuality at any time in life, especially old age, may increase risk of prostatic cancer. The data suggest that deprivation of sexuality in later life begins at about the time when incidence of prostatic cancer begins to rise.

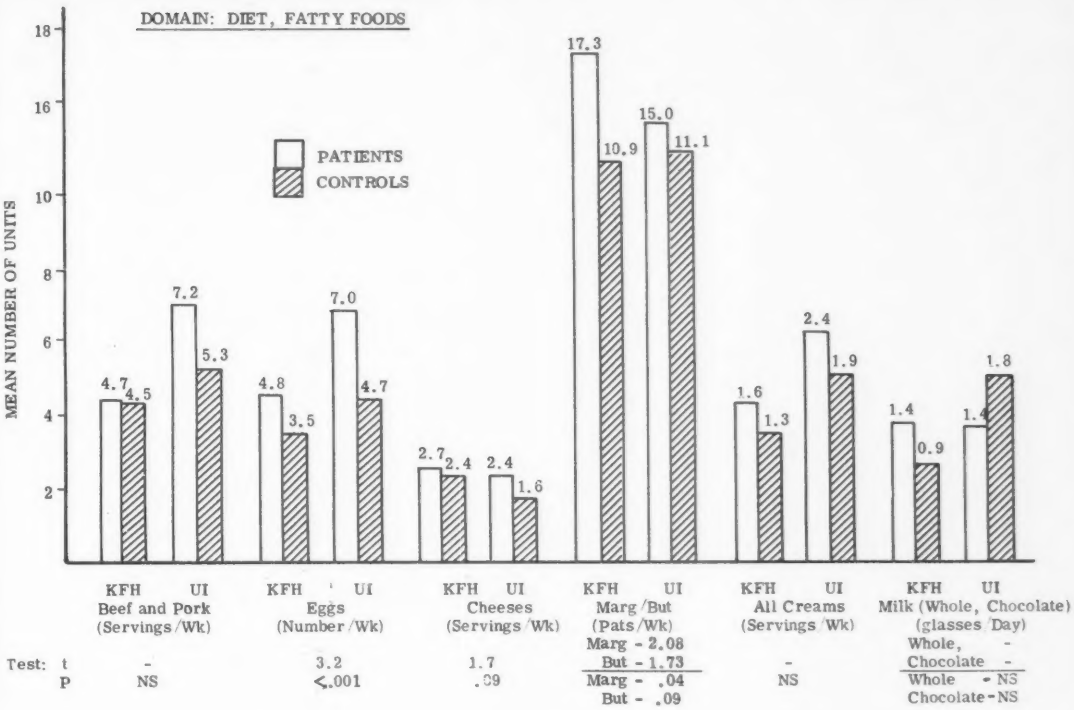

FIGURE 5.—Relative ingestion of fatty foods in the diets of prostate cancer patients and controls. KFH = Kaiser Foundation Hospital, UI = University of Illinois/West Side VA Hospital.

There is reason from the data to believe that differences between patients and controls early in life are hormonally conditioned, an impression derived from late onset of secondary sexual characteristics and delayed sexual development. Although it is possible that a hormonal rationale may also be justified for patients after 50 years of age, it is necessary to associate denial of sexuality with a culture that views sexual activity in older men as abnormal. Accumulating secretions may concentrate and have a profound effect upon the origin of neoplasia.

Excessive occupational exposures to fertilizers and auto exhaust fumes by patients, results which developed in the smaller earlier sampling, have persisted. These will be watched in the full sampling before conclusions will be reached that one or both of these variables are exogenous influences operating upon carcinogenesis in the prostate and perhaps interacting with genetic susceptibility.

There still seems to be no detectable influence of personality differences and stressful events upon the initiation or promotion of the cancer. An excess of controls experienced the effect of broken homes during adolescence, and there were no differences between patients and controls with regard to severe depressions antecedent to detection of the carcinoma.

Except for the new finding of a significant excess of patients who had been circumcised at birth, a result which is difficult to interpret and which appears to be unrelated to any other findings so far, results still oppose any hypothesis favoring transmissibility of an oncogenic or carcinogenic agent from female to male. Differences failed to develop between patients and controls for numbers of marriages, numbers of sex partners, and circumcision status. Results support more strongly than before the provisional acceptance of an endogenous initiat-

ing pathway early in life which may be hormonal, and promotion of the lesion later in life after premature cessation of sexuality, following a latent period which may be ≥ 40 years in duration.

As before, although 111 matched pairs of patients and controls provide a considerable sample compared to other studies and the original report from this study, trends and impressions should be held in abeyance until all sampling is concluded, and until data treatments have been completed.

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Epidemiologic Study of Prostatic Cancer: Preliminary Report[1]

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SUMMARY

In a preliminary report on a small fraction of the projected subject population, some evidence in support of both sexual activity and venereal transmission hypotheses is accumulating. Prostatic cancer cases, in contrast to hospitalized and/or neighborhood controls, are beginning to show greater proportions of selected sexual activities compatible with venereal transmission of an infectious agent, such as number of sexual partners, use of prostitutes, prior venereal disease, and genital infections in the spouse. Patients with prostatic cancer also appear to have had higher fertility and more prostatic cancer in blood relatives than controls. Age at first intercourse and at first marriage are lower among the cancer patients than among the controls. Antibody titrations for herpesvirus and cytomegalic virus, although currently not revealing striking disparities in positivity, tend to show higher titers among the cancer cases.

[Cancer Treat Rep 61:181–186, 1977]

It has been estimated that in 1976 there will be 56,000 new cases of carcinoma of the prostate and 19,300 deaths, thus placing this cancer second among all cancers in men in the United States (1). The Third National Cancer Survey reported a higher incidence of such cancers in the Minneapolis-St. Paul standard metropolitan statistical area than for any of the other areas involved, a rate even higher than for lung cancer, the nation's leading cancer among men (2,3). A number of mortality studies have provided demographic clues for the development of etiologic hypotheses (4–10), but only a few morbidity studies have been done (11–18).[7] Despite the magnitude of the prostatic cancer problem, few analytic epidemiologic studies entertaining specific hypotheses and based on incident cases have been initiated. The relatively higher availability of case material in the Minneapolis-St. Paul area and the paucity of epidemiologic studies in this common cancer site prompted us to entertain a more nearly definitive case-control study.

The epidemiologic evidences, though meager, do point to sexual and venereal activity hypotheses as being worthy of exploration. The sexual activity hypothesis is supported by the higher risk for prostatic cancer among married, widowed, and divorced men and among ever-married men with children (5,9). Patterns of "love-making," included in such an hypothesis, may well explain differences in incidence rates between cultures living in the same environment (19).

Certainly not unrelated to and possibly a component of a sexual activity hypothesis is an hypothesis of venereal transmission. In cervical cancer the incidence among prostitutes and the association of

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[6]The assistance of Dr. Clyde Martin, Gerontology Research Center, National Institute on Aging, in structuring our interview questionnaire and the zealous and unstinting labors of Katherine Jenkins and Evelyn Sladkey, nurse-interviewers, and Larry Edmonson and Martin Reape III, interviewers, are gratefully acknowledged.

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[7]Steele R, and Lees REM. Sexual factors in the epidemiology of cancer of the prostate. Presented at the 98th Annual APHA Meeting, 1970.

age at first coitus and number of pregnancies and sexual partners has provided clues for a venereally transmitted virus (20,21), and, recently, cervical cancer patients have been shown more frequently to have antibodies against genital herpesvirus, a venereally transmitted agent with an ability to remain latent within tissues for long periods of time (22).

Whether a parallel exists for prostatic cancer is not known. The possibility of a viral etiology was first intimated by the isolation by Centifanto et al of a herpesvirus from the genitourinary tract of a random population of men without a history of herpesvirus infection (23) and, later, the detection by the same authors of herpes particles in the prostatic cancer tissue of a single patient (24).

The epidemiologic characteristics of the disease, though pointing toward the in-depth exploration of sexual and venereal hypotheses, does not preclude the need for the study of certain other environmental factors and habit patterns such as occupational exposure and dietary items. In this current study these and other factors are being probed, but this strictly preliminary report will, for the most part, deal only with a few selected interesting or provocative variables. It must be clearly understood that none of these factors to be discussed are firm results, because, at best, they represent extremely early findings on but a fifth of the subject population.

METHODS

Case-Control Triads

Two hundred white patients with prostatic cancer from the major hospitals of the Minneapolis-St. Paul area are being collected by a combination of urologist notification and hospital surveillance techniques. The bulk of the case load is being ascertained by two trained nurse-interviewer-abstractors who routinely visit 23 hospitals weekly and have access to admission rosters, surgical schedules, and pathology reports. Permission to approach the patient is sought from the attending physician, the urologist, or both and the patient's permission for interview is sought through a brief description of the study and an approved consent form. Initially, the nurse-interviewers obtain basic demographic data and, with permission, obtain 45 ml of blood by vacutainer for clotting and a total first morning urine specimen. Every effort is made to obtain these specimens prior to surgery if possible or at least between 8 and 10 AM. Specimen collection occasionally must be done in the home and, when necessary, a notation to this effect is made. Following a reasonable post-surgical interval, appointments for the full-scale in-depth interview are made for the male interviewers.

Having ascertained the cancer case, the nurse-interviewers select a hospitalized control in the same hospital, avoiding patients admitted for other genitourinary problems and matching the control by age (± 3 years), race, and similar admission date. The same specimen collection and interview procedures are applied to these controls.

Similarly, to control the socioeconomic factors and because hospital controls may interject selection bias for the factors under study, a second control is selected from the neighborhood of the case by mail, utilizing reverse phone directories, followed by telephone contact for the participation of such a control matched by age (± 5 years) and race. The neighborhood control is sought on the same street of residence as the case, with systematic progression north then south or east then west of the residence of the case until a suitable control is found. The same appointment, specimen collection, and interview procedures are applied as above. Thus, matched triads will be available for analyses.

Specimen Processing

The clotted blood specimens are brought to the laboratory for separation of the sera and partitioning into aliquots for several determinations. The morning urine specimen is measured and two aliquots of 100 ml each are stored in polyethylene bottles; both these and the sera are deep frozen at −80°C for storage. Small quantities of the sera are transferred in batches to the laboratory of Dr. Henry Bauer where herpesvirus and cytomegalic virus antibody determinations are made. Similarly, aliquots will be supplied to the laboratories of the National Center for Disease Control for Epstein-Barr virus antibody titrations. The balance of the sera and urines is stored for future hormonal assay in the hope of determining differences in endocrine patterns between prostatic cancer cases and controls as potential determinants of risk and as screening tools.

Interviews

Male interviewers utilize a structured interview schedule developed with the assistance of Dr. Clyde Martin, formerly of the Kinsey team. An in-depth sensitive approach is utilized by two well-trained interviewers who, in addition to obtaining residential, occupational, and medical and hospitalization histories, probe into smoking and alcohol consumption, use of drugs and medications, marital history,

TABLE 1.—Selected variables among prostate cancer cases, hospitalized controls, and neighborhood controls: preliminary results (Minneapolis-St. Paul standard metropolitan statistical area, 1976)

Variables	Prostatic cancer cases		Hospitalized controls		Neighborhood controls	
	No.	Average	No.	Average	No.	Average
Median age	40	64	43	64	35	66
Residence ever on farm	16/40	40.0%	24/43	55.8%	21/35	60.0%
Prostatic cancer in family	6/36	16.7%	3/41	7.3%	0/35	0.0
History of cirrhosis	1/40	2.5%	4/43	9.3%	0/35	0.0
History of hepatitis	2/40	5.0%	3/42	7.1%	5/35	14.3%
Ever used tobacco	37/40	92.5%	38/43	88.4%	24/35	68.6%
Ever smoked cigarettes	33/40	82.5%	37/43	87.0%	22/35	63.9%
Alcohol use	39/40	97.5%	41/43	95.3%	33/35	94.3%
Median age at first intercourse	39	18.5	42	19.0	35	20.0
Median age at first marriage	37	24.0	42	26.0	35	26.0
Median No. of children	34	3.2	34	2.6	33	2.4
Maximum intercourse in 1 wk	36	4.0	42	5.5	35	7.0
Frequency of intercourse in first yr of marriage (median per wk)	35	3.2	39	4.1	35	4.1
Median No. of sexual partners	40	4.0	42	7.35	35	3.0
Ever used prostitutes	15/40	37.5%	12/42	28.6%	10/35	28.9%
Ever had venereal disease	5/40	12.5%	2/43	4.7%	1/35	2.9%
Genital infections in wife	6/36	16.7%	10/40	25.0%	2/35	5.7%

and detailed patterns of sexual behavior and exposures. These latter questions are designed to distinguish among several patterns of sexual activity if they can discriminate between cases and controls and to provide, along with the designated antibody determinations for candidate viruses, information on possible venereal exposures.

Dietary Questionnaire

A mailed questionnaire on frequency of use of selected dietary items, developed and used successfully in our studies on gastrointestinal cancers, will be applied to all cases and controls.

RESULTS AND DISCUSSION

As of this writing 53 cancer patients, 52 hospitalized controls, and 41 neighborhood controls have had blood and urine specimens collected and 40 cancer patients, 43 hospitalized controls, and 35 neighborhood controls have been interviewed. Because of the small size of the completed sample, matched triad analyses will not be presented in this report. Contrasts or similarities will be based on group characteristics. The number of cases and controls are too few to perform any meaningful cross-tabulations.

Table 1 presents selected characteristics relevant to sexual or venereal hypotheses as well as several other considerations. It should be noted that even without matched-pair analysis the median ages of the case and control groups are virtually identical, indicating reasonably good age-matching procedures. The residential history of the subjects, even with this small size, reveals that fewer prostatic cancer cases than either type of control ever lived on a farm. Although Lilienfeld et al (4) found no urban/rural differences in age-adjusted mortality rates for whites in the United States, King, Diamond, and Lilienfeld had earlier examined the mortality data for metropolitan and non-metropolitan counties by census regions and had found an excess among white residents of metropolitan counties in all but the northeastern region of the country (5). Our data on living subjects thus far are compatible with the latter finding. This would also be compatible with the observation that prostatic cancer occurs less frequently among agricultural workers (5).

Relatively few studies of familial aggregation of cancer of the prostate have been done. Most significant is the one by Woolf among Mormons in Utah in which there was an excess of prostatic cancer mortality among male relatives of prostatic cancer index cases (25). Of immediate interest in our study, despite the small numbers collected thus far, is the

finding of an excess of prostatic cancer cases with a history of prostatic cancer in at least one member of the family. Six of the cases (16.7%) revealed cancer of this site as compared to none among 35 neighborhood controls. Of the six cases, four had fathers with prostatic cancer, one had a brother, and one had a grandfather with the disease. One of the four who had a father with this cancer also had two maternal uncles with the disease. Our finding is thus compatible with that of Krain (13) and Steele and Lees* who found similar results but also with very small numbers. However, three of the 41 hospitalized controls (7.3%) also indicated that their fathers had had prostatic cancer.

There are an insufficient number of subjects in the study so far to examine a number of other sites of cancer including breast cancer among the female blood relatives. Thiessen (26) found a significantly higher incidence of prostatic cancer among male relatives of breast cancer patients. Wynder et al (27) have indicated a positive correlation between prostatic and breast cancer in a study of international mortality. We are currently collecting data to ascertain whether an excess of breast cancer does exist among the female blood relatives of our subjects as clues to either a common genetic or environmental component for these potentially hormone-dependent cancers.

The hypothesis that cirrhosis of the liver "protects" against prostatic carcinoma is intriguing and is suggested by Robson (28). This hypothesis is compatible with the observed relative mortalities among whites and blacks in the United States in the age groups of greatest mortality from prostatic cancer. The inverse relationship of rates for these two diseases in a country such as Denmark also fits this hypothesis. A biologic mechanism for this relationship may exist in the observation that hepatic cirrhotics, produced either by alcohol or malnutrition or both, have higher than normal estrogen levels (28). In an autopsy study by Glantz a significantly lower proportion of such cirrhotics had evidence of prostatic cancer than did a control group. He suggested the protective effect of hyperestrogenism (29). We are attempting to test this relationship by history of cirrhosis. In table 1 it is obvious that an adequate denominator of subjects does not yet exist. For whatever it may mean, the four cirrhotics among the 43 hospitalized controls are not surprising since this disease is not an exclusion criterion in the selection of matched controls.

Although without apparent relationship between hepatitis (unspecified) and cirrhosis there also are fewer prostatic cancer cases with a history of hepatitis than hospitalized controls and even less than in neighborhood controls. This finding, if maintained to the conclusion of the study, could indicate that altered liver metabolism may be related to prostatic cancer.

With the accumulation of subjects, we shall be able to subdivide for current and discontinued smoking and types and amounts thereof. Of interest at this time is the marked disparity in the proportions designating any use of tobacco between the neighborhood controls (68.6%) and both the cases (92.5%) and hospitalized controls (88.4%). The same general disparities are noted for cigarette smoking. Not only may the similarity of proportions between the cases and the hospital control groups be a reflection of the well-documented greater morbidity rates among smokers than non-smokers (30), but it points up the hazard of utilization of hospital controls for variables which may be related to a number of diseases or to morbidity in general. The merit of a neighborhood control over a hospitalized control is readily apparent here.

Alcohol consumption is also under scrutiny and although "use" data are at this time non-discriminating, frequency and amount of use will be analyzed later.

In structuring an approach to the major hypotheses, namely, types and modes of sexual activity and venereal transmission, a number of variables were considered. Some of these variables could be deemed to be indicators of frequency or intensity and span of sexual activity such as age at first intercourse, age at first marriage, number of marriages, fertility as measured by number of children claimed, maximum times intercourse was performed in any 1 week in the subject's life, number of sexual partners, intercourse with prostitutes, etc. Some of these, such as age at first intercourse, frequency of intercourse, number of sexual partners, use of prostitutes, as well as evidence of venereal disease, genital infections in the wife, types of intercourse or sexual play (such as homosexual encounters), oral or anal sex, and herpesvirus 2 antibody levels, could be indicators of venereal transmission. In addition, the styles of love making, arousal with and without fulfillment, and types and duration of foreplay are also being explored. Time and the small sample size permit only limited analyses of a few of these variables.

In table 1 the median age at which first intercourse was experienced was lower for the prostatic cancer cases (18.5 years) than for either control group (19.0 and 20.0 years). Furthermore, the median age at first marriage was also lower for the cancer cases (24.0 years) than for either control group (26.0 years).

*See footnote 7.

TABLE 2.—Distribution of serum antibody titers for herpesvirus and cytomegalic viruses among prostatic cancer cases, hospitalized controls, and neighborhood controls: preliminary results (Minneapolis-St. Paul, 1976)

	Cases		Hospitalized controls		Neighborhood controls	
Titers	No.	%	No.	%	No.	%
Herpesvirus						
≤ 1:8	28	52.8	33	63.5	17	41.5
1:16	5	9.4	9	17.3	10	24.4
1:32	14	26.4	6	11.5	11	26.8
1:64	6	11.3	3	5.8	3	7.3
1:128	0	0.0	1	1.9	0	0.0
Total	53	99.9	52	100.0	41	100.0
Cytomegalic virus						
≤ 1:8	23	43.4	22	42.3	21	51.2
1:16	6	11.3	8	15.4	6	14.6
1:32	10	18.9	13	25.0	10	24.4
1:64	13	24.5	7	13.5	3	7.3
1:128	1	1.9	2	3.8	1	2.4
Total	53	100.0	52	100.0	41	99.9

The ever-married prostatic cancer cases had a greater average number of children (3.2) than either of the two ever-married control groups (2.6 and 2.4). This tends to confirm the findings of Armenian et al with respect to fertility among prostatic cancer cases (15). Also, Greenwald et al reported that fewer prostatic cancer cases than controls had no children (8).

Prostatic cancer cases claimed a smaller median maximum number of times intercourse was performed in any 1 week of their lives (4.0) as compared to either control group (5.5 and 7.0). This finding is compatible with that of Steele and Lees[9] who found that more of the controls than cases reported having more than three coital experiences during the week of maximum activity.

Also, in response to the question "During the first year of your marriage, what was the customary frequency of intercourse?", the median number reported by the cases (3.2) was less than those for either control group (4.1). Both of these questions, according to Martin, correlate well with sexual activity (personal communication). In fact, based on his research, he found that the latter question was the best indicator of "sex drive" or quantity of ejaculatory performance. This is consistent with an hypothesis of stimulation without fruition. In fact from Steele's study[9] information exists that prostatic cancer cases experienced "greater sexual drive estimated in terms of interest in more intercourse than occurred" than did controls.

[9]*See* footnote 7.

A number of variables might indicate venereal transmission; however, some of these may be more significant (eg, visiting prostitutes and a history of venereal disease) than others (eg, number of sexual partners and wives' genital infections). Thus, although the modest excess in the median number of sexual partners (table 1) and the excess of genital infections among the wives of prostatic cancer cases is seen only in relation to the neighborhood controls, a greater proportion of cases (37.5%) than of either control group (28.6% and 28.9%) visited prostitutes and a greater proportion had had venereal diseases (12.5% vs 4.7% and 2.9%).

Studies of the proportions of subjects with antibody against herpesvirus 2, cytomegalic virus, and Epstein-Barr virus as candidates for a viral etiology in prostatic cancer are also under way as indicated above. Preliminary results with herpesvirus and cytomegalic virus are presented in table 2. There appear to be higher proportions of higher titered sera for both herpesvirus and cytomegalic virus antibodies among the cases than among either of the two sets of controls. Quality control has been instituted utilizing blind replicates. We are currently arranging for other herpesvirus 2 antibody tests according to the methods utilized by Aurelian or Rawls or both.

CONCLUSIONS

This is an interim report with several interesting early findings in regard to both sexual activity and venereal transmission hypotheses. Whether the trends noted in a number of the designated varia-

bles will assume statistical significance with an increase in sample size remains to be seen. With an increase in the case and control load under study, other variables, particularly with respect to modes of sexual activity, will lend themselves to analysis, and cross-tabulations among confounding and interacting variables will become possible.

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Race, Socioeconomic Status, and Prostatic Cancer [1]

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SUMMARY

Using mortality and incidence data from Alameda County, California, this study attempted to determine whether the higher occurrence rate of prostatic cancer among black men as compared with whites in the United States might be explained by racial differences in factors associated with socioeconomic status. Each death or case of prostatic cancer was assigned to a social class based on census tract of residence, and rates by race and socioeconomic status were computed. Comparison of age-specific mortality and incidence rates by socioeconomic status reveals no gradient in either whites or blacks. The higher risk for blacks holds up at almost every age and socioeconomic level. However, the racial differences are less pronounced for incidence than for mortality. Racial differences in the occurrence of deaths appearing in Part II of the death certificate are also examined.

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Two facts concerning the epidemiology of prostatic cancer are outstanding: (*a*) the very strong positive association of this cancer with age, and (*b*) the sizable higher occurrence rate of the disease among black men as compared with whites in the United States. It has been our objective to explore some possible social/environmental correlates of this association of prostatic cancer with race, using mortality and incidence data from the San Francisco Bay Area.

Several investigators have previously attempted to determine whether there is an association of prostatic cancer with socioeconomic status, but the results reveal no consensus (1–11). One frequently quoted earlier review tentatively concludes that there is a higher frequency of the disease among professionals than among other classes of workers (7). If it is true that professional workers are at increased risk for prostatic cancer, how does one explain the higher black rates for the disease in the United States, since blacks comprise a disproportionately large part of the lower economic classes? Do black/white differences in the occurrence of this cancer hold up when social class is controlled? These questions prompted the current study. Using population-based mortality and incidence data, each death or case of prostatic cancer was assigned to a social class based on census tract of residence, and rates by race and socioeconomic status were compared.

DATA AND METHODS

The study population was that of Alameda County in northern California. Mortality data were obtained for the two pericensual periods 1958–62 and 1968–72. During the first period, a total of 341 deaths occurred in which prostatic cancer was listed as the primary cause (Part I of the death certificate), and another 126 occurred for which it was listed as an "other significant condition" related to death (Part II). For 1968–72, there were 395 Part I and 199 Part II deaths. Incidence data were drawn from the complete incidence series in the population-based Alameda County Cancer Registry which spanned the years 1960–69. Cases among whites and blacks for the 10-year period numbered 2197.

Census tracts were grouped into four socioeconomic levels based on two indicators: education and

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TABLE 1.—Average annual age-specific prostatic cancer mortality rates by race and socioeconomic status: Alameda County, 1968–72

Age group and socioeconomic status*	Whites in all tracts (1)	Whites in "black tracts" (2)	Blacks in "black tracts" (3)	Black/white ratio (3/1)
50–59 yrs				
I (low)	6.3	18.3	22.8	3.6
II	7.8	12.4	27.4	3.5
III	10.6	25.3	0.0	—
IV (high)	2.8	0.0	—†	—
Total	7.4	14.4	22.9	3.1
60–64 yrs				
I	30.2	16.9	96.8	3.2
II	46.5	37.4	21.2	0.46
III	17.9	0.0	0.0	—
IV	6.8	0.0	—	—
Total	30.7	22.3	60.3	2.0
65–69 yrs				
I	41.0	60.9	171.7	4.2
II	54.4	31.5	170.9	3.1
III	64.5	0.0	—	—
IV	36.7	60.2	—	—
Total	51.0	41.0	163.9	3.2
70–74 yrs				
I	101.8	53.4	396.0	3.9
II	139.6	138.5	341.9	2.4
III	70.1	0.0	—	—
IV	79.8	69.9	—	—
Total	106.2	84.3	376.2	3.5
75+ yrs				
I	308.2	244.3	353.4	1.1
II	322.8	359.2	561.8	1.7
III	337.9	371.1	—	—
IV	363.3	490.6	—	—
Total	331.9	343.4	408.6	1.2

*Defined here as the % of individuals in the tract 25 yrs and older with some college education: I = 0–14%; II = 15%–29%; III = 30%–44%; IV = ≥ 45%.
†— = insufficient numbers.

income. In census tracts where 400 or more non-whites reside, referred to here as "black tracts," socioeconomic status indicators are available for non-whites as well as for the total population. Preliminary comparisons on a variety of social indicators revealed that, in every instance, non-whites in such tracts were slightly disadvantaged in comparison with whites in the same tracts, thus indicating that the two racial groups may live in the same neighborhood but not enjoy the same social status. Therefore, the black mortality rates examined here are based on those tracts for which non-white socioeconomic status information could be so obtained.

RESULTS

Mortality

Comparison of the age-specific Part I mortality rates by socioeconomic status reveals no gradient for prostatic cancer in either whites or blacks. This is illustrated in table 1 for Alameda County (1968–72), using percent college-educated as the socioeconomic indicator. The higher risk for blacks holds up at almost every socioeconomic level. The summarized ratio of blacks to whites is 2.3 in Social Class I and 2.0 in Social Class II.

For both pericensual periods, there was an impressive clustering of black deaths in black tracts and a lower than expected number of deaths among blacks residing in predominantly white areas. In 1968–72, for example, only one death was observed outside of black tracts. Given the age distribution of blacks in these areas, the expected number of deaths based on rates within black tracts would have been 4.3. This finding, although based on small numbers, might point to a cultural or environmental factor(s) not tapped by our indirect measure of social class.

When Part II deaths are also considered, certain

TABLE 2.—Comparison of Part I/Part II deaths from prostatic cancer by race and time period: Alameda County, 1958–62 and 1968–72

	Prostatic cancer listed in—			Part II/Part I ratio
	Part I	Part II	Total	
	1958–62			
Whites	305	119	424	0.39
Blacks	36	7	43	0.19
	1968–72			
Whites	326	174	500	0.53
Blacks	69	25	94	0.36

TABLE 3.—Age distribution of Part I and Part II deaths from prostatic cancer among white males: Alameda County, 1968–72

Age	Prostatic cancer listed in—		Part II/Part I ratio
	Part I	Part II	
50–59	16	1	0.06
60–64	25	5	0.20
65–69	30	12	0.40
70–74	47	26	0.55
75+	208	130	0.63
Total	326	174	0.53[1]

racial differences and changes over time in the ratio of Part I to Part II deaths are noteworthy. As is shown in table 2, in both races the likelihood of a prostatic cancer death being listed in Part II increased from 1960 to 1970. For both pericensual periods, blacks were considerably less likely than whites to have their prostatic cancer listed in Part II, perhaps indicating several things: (*a*) blacks may have more severe prostatic cancer and therefore be likelier to have it listed in Part I; (*b*) since whites with prostatic cancer may have higher survival rates than blacks, it could be that the course of their disease has a greater chance of being interrupted by another cause of death; and (*c*) whites may receive better medical screening and have a greater opportunity for early-stage prostatic cancer diagnosis.

For whites in Alameda County during the period 1968–72, there is a clear positive age gradient in the probability of prostatic cancer appearing in Part II, as shown in table 3. A man under the age of 65 dying with prostatic cancer is four times more likely to have his cancer listed as the primary cause of death than is his counterpart over the age of 75. This agrees with the well-documented observation from autopsy series that the incidence of latent carcinoma of the prostate increases dramatically with age and that a substantial proportion of older men in the general population are likely to die with, but not of, the disease (12).

Incidence

Table 4 shows annual age-specific incidence rates by race and socioeconomic status for Alameda County (1960–69). As was true for mortality, there is no evidence of an association of prostatic cancer incidence with social class in either whites or blacks. It is interesting to note, however, that the ratios of black to white incidence rates at most age and socioeconomic levels are considerably below those observed for mortality rates. Also, the black/white ratio appears to be higher in men below 65 as compared with those above 65. The reduced racial differential emphasizes the fact that mortality rates alone cannot always be used as a gauge of racial risk factors in the occurrence of disease. The assumption that death rates accurately reflect relative differences, even though at lower absolute levels, may be mistaken. That Part II deaths were more common in whites than blacks indicated that this might be the case.

Incidence rates are higher than mortality rates in every age group, as shown in table 5. Among whites, there are about four cases of prostatic cancer for every death in men under the age of 75, but this ratio declines to 2.2 cases per death for men aged 75 and older. One might have expected the opposite, given the rising percentage of latent prostatic cancer with advancing age. Of course, the increased mortality in the older age groups is occurring in part among men who became incident cases at younger ages. This age pattern is not borne out in blacks. Moreover, the case-to-death ratio is much lower for blacks than whites, which could be indicative of a higher case fatality.

DISCUSSION

Two possible biases in prostatic cancer reporting should be mentioned. The first is census underenumeration, known to operate more for blacks than whites, which may decrease black denominators and

TABLE 4.—Average annual age-specific prostatic cancer incidence rates by race and socioeconomic status: Alameda County, 1960–69

Age group and socioeconomic status*	Whites (1)	Blacks† (2)	Black/white ratio (2/1)
50–59 yrs			
I	49.9	83.3	1.7
II	26.5	48.2	1.8
III	22.8	50.8	2.2
IV	37.0	42.6	1.2
Total	30.4	62.4	2.1
60–64 yrs			
I	131.2	216.5	1.7
II	108.7	202.8	1.9
III	159.2	300.0	1.9
IV	63.3	63.3	1.0
Total	114.3	207.4	1.8
65–69 yrs			
I	310.0	353.9	1.1
II	355.6	276.4	1.1
III	241.9	344.8	1.4
IV	254.1	—‡	—
Total	261.2	315.6	1.2
70–74 yrs			
I	527.4	654.4	1.2
II	431.4	362.0	0.8
III	441.6	—	—
IV	402.0	—	—
Total	445.1	517.9	1.2
75+ yrs			
I	689.4	1042.7	1.5
II	676.6	836.7	1.2
III	786.3	—	—
IV	582.0	—	—
Total	681.3	845.9	1.2

*See table 1 for definition.

†Due to problems inherent in interpolating race- and age-specific populations at the census tract level for intercensual years, in particular the fact that black-specific figures were not available before the 1970 census, the population denominators employed here are those for 1970. The net effect of using 1970 rather than 1965 populations is to slightly depress actual rates, although relative differences between the races should be approximated. Also, for the computation of incidence rates, blacks and whites residing in the same census tract were assigned the same socioeconomic level.

‡— = insufficient numbers.

TABLE 5.—Comparison of age-specific annual incidence rates for 1960–69 with mortality rates for 1968–72: Alameda County

Age	Incidence rates	Mortality rates	Incidence/ mortality ratio
	Whites		
50–59	30.4	7.4	4.1
60–64	114.3	30.7	3.7
65–69	261.2	51.0	5.1
70–74	445.1	106.2	4.2
75+	681.3	331.9	2.1
	Blacks		
50–59	62.4	22.9	2.7
60–64	207.4	60.3	3.4
65–69	315.6	163.9	1.9
70–74	517.9	376.2	1.4
75+	845.9	408.6	2.1

therefore artificially increase black disease rates. This caveat is advanced cautiously, because it appears that black/white differences in underenumeration are least in older age groups, ie, those at greatest risk for prostatic cancer. The other potential source of bias relates to medical screening which may be more efficient for whites. This detection bias operates, of course, for all cancers but might be especially critical in prostatic cancer, known to frequently occur in subclinical form. In brief, then, census underenumeration could serve to increase black rates while less routine medical screening could serve to decrease them.

Since this study relied upon indirect indicators of socioeconomic status, the group averages may or may not represent the experience of the individual under study. One might imagine that this potential shortcoming of census data is more pronounced for diseases striking the very old than the young, since the latter contribute in larger numbers to determining census tract values. How these factors might operate to diminish any potential differences in rates between the social classes or how they might affect the races differentially is unclear.

We are left with a basic "misfit" in the epidemiologic evidence on prostatic cancer. It has variously been called a disease of professionals and westernization, and it is expected to have a greater likelihood of diagnosis in men receiving regular examinations. Thus, the sizable higher occurrence rate for blacks in the United States is unexpected. Also surprising is the lack of an association of the disease with socioeconomic status demonstrated here. We now intend to focus on additional cultural/environmental and genetic factors which might account for the still unexplained racial differences in the occurrence of this increasingly important cancer.

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Enzyme Markers in Human Prostatic Carcinoma [1]

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SUMMARY

In an effort to develop a sensitive and specific method for detecting human prostatic cancer at early stages, we have studied the isoenzyme patterns of acid phosphatase in patients' sera as well as in benign hypertrophic and cancerous prostatic tissues using isoelectric focusing techniques. At least eight acid phosphatase isoenzymes at pI 4.1–5.5 could be observed. The sera with highly elevated acid phosphatase activity generally contained more isoenzymes with pI values of 4.5–5.0. The purified acid phosphatase isolated from benign hypertrophic and malignant prostatic tissues showed no qualitative difference in isoenzyme patterns although quantitative variations were observed. Malignant tissue contained more isoenzymes with pI values of 4.5–4.8. Patients' sera were found to contain isoenzymes of prostate origin. We have also investigated serum ribonuclease (RNase) activity in patients with prostatic cancer. The serum RNase activity of patients was significantly elevated. No significant correlation was observed between serum acid phosphatase and RNase activity. In some instances, where acid phosphatase activity was in the normal range, RNase activity was elevated. These data suggest that simultaneous measurements of RNase and acid phosphatase activities may be of value in the diagnosis of prostatic cancer. The purified RNase has been isolated from human prostatic tissue and its immunologic properties are being studied.

[Cancer Treat Rep 61:193–200, 1977]

The success of cancer treatment generally depends upon the early detection of disease. Therefore, we have been interested in serum enzymes of prostatic carcinoma that can be used as tumor markers for early diagnosis. During the course of prostatic cancer, a number of biochemical changes in serum occur. One such change, the elevation of serum acid phosphatase activity, has been extensively studied as an aid for diagnosis (1). However, the measurement of serum acid phosphatase by conventional spectrophotometric methods often fails to detect prostatic cancer at its early stages. The acid phosphatase exists in more than one isoenzyme form, having characteristic properties and tissue specificity (2). It should be possible to develop a sensitive and specific method for detecting minute amounts of serum acid phosphatase of prostatic origin during early stages of the disease. Consequently, we have studied the isoenzyme patterns of acid phosphatase in serum and in benign hypertrophic as well as malignant prostatic tissues using an isoelectric focusing technique.

We have also examined the ribonuclease (RNase) activity in the serum of patients with prostatic tumor in view of the reports that serum RNase activity increases in patients with various types of neoplasms (3). Isolation of an RNase from prostatic glands and preparation and partial characterization of antiserum to this enzyme are presented in this paper.

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MATERIALS AND METHODS

Materials

Benign hypertrophic and malignant prostatic tissues collected during surgery and autopsy were obtained from this Institute and local hospitals, as well as from the University of Miami, National Prostatic Cancer Project (NPCP) Tissue Bank. Tissues were stored at −75°C immediately until used. Serum samples from patients with histologically proven carcinoma of the prostate were furnished by this Institute and by the NPCP Chemotherapy participating institutes.[4]

Neuraminidase, α-naphthyl phosphate, fast Garnet GBC salt, fast red salt B, and bovine pancreatic RNase A were obtained from Sigma Chemical Co (St. Louis, Mo). Materials used in isoelectric focusing such as ampholyte (pH 4–6), acrylamide, etc, were purchased from Bio-Rad Laboratories (Richmond, Calif) and agarose: 5′-(*p*-aminophenylphosphoryl) uridine 2′(3′) phosphate was purchased from Miles Laboratories (Elkhart, Ind).

Ascites fluid from a patient with pancreatic carcinoma was obtained from this institute and was extracted by Dr. S. Harvey of our department with perchloric acid (PCA) according to Reddi's procedure (4). This PCA extract contained 0.4 mg/ml of protein and 760 units (*see* below) of pancreatic RNase activity.

Crude Extract of Tissues

Prostatic tissues were weighed, minced, and homogenized in 3 volumes of 0.01% Tween 80 in 0.02 M sodium acetate buffer, pH 5.2. The homogenized material was stirred at 4°C overnight, followed by centrifugation at 10,000 × *g* for 20 minutes (RC2-B Superspeed Centrifuge, Sorvall). The supernatant was dialyzed overnight against the same buffer.

Purification of RNase

The hypertrophic prostatic tissues (130 g) were extracted with Tween 80 as described above. The crude extract thus obtained was applied on the agarose: 5′-(*p*-aminophenylphosphoryl) uridine 2′(3′) phosphate column and the RNase which retained on the column was eluted by convex gradient of 0.02 M acetate buffer, pH 5.2 (150 ml)-0.02 M acetate buffer, pH 5.2, containing 50 mM sodium phosphate (300 ml). Fractions containing RNase activity were pooled and dialyzed against tris-HCl buffer, pH 7.5, which contained 0.1 M NaCl. The dialyzed enzyme solution was further applied on CM-Sephadex column (2.5 × 35 cm) which had been equilibrated with the same buffer. The column was first washed with 300 ml of the aforementioned buffer. RNase was then eluted from column by convex gradient of 0.05 M tris-HCl, pH 7.5 (150 ml)-0.5 M tris-HCl, pH 7.5, containing 0.5 M NaCl (300 ml).

Enzyme Activities

Acid phosphatase activity was measured by the method of Babson and Phillips (5) using α-naphthyl phosphate as substrate. The hydrolyzed product, α-naphthol, formed a stable colored complex with fast red salt B in alkaline condition and the absorbance at 588 nm was measured. One international unit (IU) of acid phosphatase activity was defined as the amount of enzyme in 1 liter of sample which will hydrolyze the substance at a rate of 1 μmol/minute. Acid phosphatase activities in sera of normal men were 1.0–4.0 IU.

RNase activity was determined by Shapira's method (6). Briefly, this method utilized thionine-RNA complex as substrate and determined the decrease of OD_{625} of substrate (Beckman DB-G spectrophotometer). One unit of RNase activity was defined as the amount of RNase in 1 liter of sample that will cause the decrease of OD_{625} of reaction mixture at a rate of 1 OD unit/minute. The normal ranges of serum RNase were 92–296 with this procedure.

Treatment of Neuraminidase

The reaction mixture, containing 40 μl of serum sample, 0.005 mol of acetate buffer, pH 5.0, and 0.002 mg protein (0.001 units) of neuraminidase in a final volume of 50 μl was incubated at room temperature for 20 hours. The mixture was then used as it was in isoelectric focusing.

Isoelectric Focusing

This was carried out at 4°C using Shandon Southern analytic polyacrylamide gel electrophoresis apparatus. Polyacrylamide gels (5 × 70 mm) were prepared according to procedures described in the instruction sheet from the Bio-Rad Laboratories. Phosphoric acid (0.02 M) was used as the anolyte and NaOH (0.01 M) as the catholyte. Samples were mixed with an equal volume of 50% sucrose solution and 50 μl of sample sucrose solution was applied on top of the gels (anode side). A constant voltage of 120

[4]NPCP Chemotherapy participating institutes: Virginia Mason Research Center, Seattle, Wash; M. D. Anderson Hospital and Tumor Institute, Houston, Tex; University of Iowa Hospitals and Clinics, Iowa City; Massachusetts General Hospital, Boston; and Johns Hopkins Hospital, Baltimore, Md.

volts was applied for 20 hours. After electrophoresis gels were stained for acid phosphatase activity using 0.1% α-naphthyl phosphate-0.1% fast Garnet GBC salt in 0.1 M ammonium acetate buffer at pH 5.0 (7).

Preparation of Anti-RNase Serum

Purified prostatic RNase (100 μg protein/ml) was mixed with an equal volume of complete Freund adjuvant and 1 ml of this emulsified mixture was injected sc to the back of each female rabbit. After 4 weeks four subsequent booster injections at the same dosage as the primary immunization were made at 2-week intervals. Sera were then collected 2 weeks after the last booster injection.

Absorption of antiserum with normal female serum (NFS) was carried out by mixing antiserum with equal volume of NFS and incubating at 37°C for 30 minutes, then at 4°C for 3 days. Absorption of the NFS-treated antiserum with PCA extract of ascites fluid from the patient with pancreatic cancer was carried out as described above.

Ouchterlony's Double Immunodiffusion

Melted 1% agarose in 0.15 M phosphate buffer (8) was pipeted (8 ml) into a Petri dish with a diameter of 8 cm. After gelatinization, wells were punched out with a No. 2 borer. To each well 20 μl of antiserum or antigen was added. After 18 hours in a moisturized chamber, the gels were washed with 0.154 M saline for 3 days and then were stained for protein with Coomassie solution (9).

Determination of Protein Concentration

The procedure of Lowry et al (10) was used. Bovine serum albumin was used as the standard.

RESULTS AND DISCUSSION

Isoenzymes of Acid Phosphatase

Many isoenzyme bands of acid phosphatase were observed on gel column when the sera of patients with prostatic carcinoma were subjected to isoelectric focusing. The patterns of acid phosphatase isoenzymes varied from patient to patient. There was a tendency for sera with highly elevated acid phosphatase activity to have more isoenzymes with pI values of 4.5–5.0. Figure 1 shows a representation of these observations. It can be seen that the serum with acid phosphatase activity of 3700 IU had more isoenzymes with pI 4.5–5.0 than other serum samples. Serum with a slightly elevated acid phosphatase activity (14.7 IU) contained more isoenzymes at pI values of 5.0–5.5.

These isoenzymes of serum acid phosphatase may represent various molecular isomers with different contents of neuraminic acid as with those in human prostatic gland (9). As shown in figure 2, treatment of serum specimen with neuraminidase to remove sialic acids from the enzyme resulted in converting the original eight isoenzyme bands into one broad and one narrow band which migrated at a higher pH region.

In order to study whether there are any acid phosphatase isoenzymes which are unique to malignant prostatic tissue, crude extracts of benign hypertrophic and malignant prostatic tissues were subjected to isoelectric focusing. No qualitative difference in isoenzyme patterns was revealed between the extracts prepared from these two tissues, but some quantitative variations were observed. The malignant tissue contained more isoenzymes with a pI value of 4.5–4.8 (fig 3). In addition, both benign hypertrophic and malignant tissues contained the isoenzymes, which could be found in patients' sera, indicating the prostatic origin of serum acid phosphatase.

Experiments with serum from normal, healthy individuals revealed that no acid phosphatase isoenzyme bands were detected by isoelectric focusing. Furthermore, serum specimens from patients with Gaucher's disease, with elevated acid phosphatase activities (16.9 IU) by conventional spectrophotometric method, revealed a broad diffuse isoenzyme band at around pI 5.0, which was different from those of prostatic origin.

These results indicated that there was a difference in serum acid phosphatase at the molecular level from patients with prostatic carcinoma and normal men or patients with other diseases. It is, therefore, probable that specific antiserum can be produced to the prostatic acid phosphatase isoenzymes for a better detection of this neoplasm.

Serum RNase Activity of Prostatic Carcinoma

Table 1 shows that serum RNase activities from patients with prostatic carcinoma were significantly higher ($P < 0.01$) than those from normal healthy men. No correlation was observed between serum RNase and acid phosphatase activities. In some instances where acid phosphatase activities were within normal range (< 4.0 IU), RNase activities were elevated. These results suggest that the simultaneous determinations of serum RNase and acid phosphatase activities may be of potential value in diagnosis of prostatic cancer, since the combined positive detection rate increased to 85% from 71%

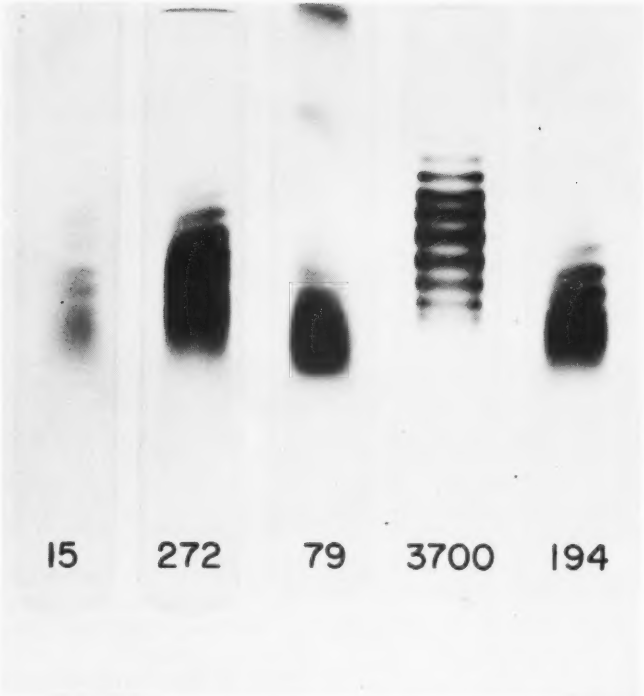

FIGURE 1.—Isoenzyme patterns of serum acid phosphatase from patients with prostatic carcinoma. The figures at the bottom of each tube represent the enzyme activities (in IU) measured by conventional spectrophotometric method. The isoelectric focusing was performed between pH 4.0 (top of gel) and 6.0 (bottom of gel).

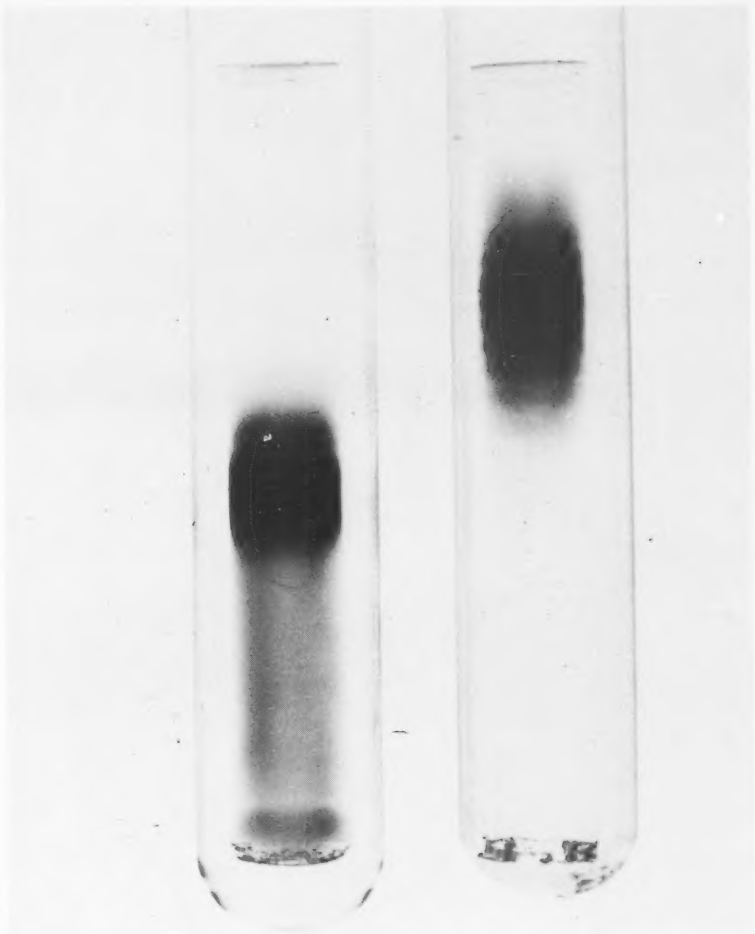

FIGURE 2.—Acid phosphatase isoenzyme patterns from a serum with 191 IU of enzyme activity before (tube at the right) and after (left) treatment with neuraminidase. (*See* fig 1.)

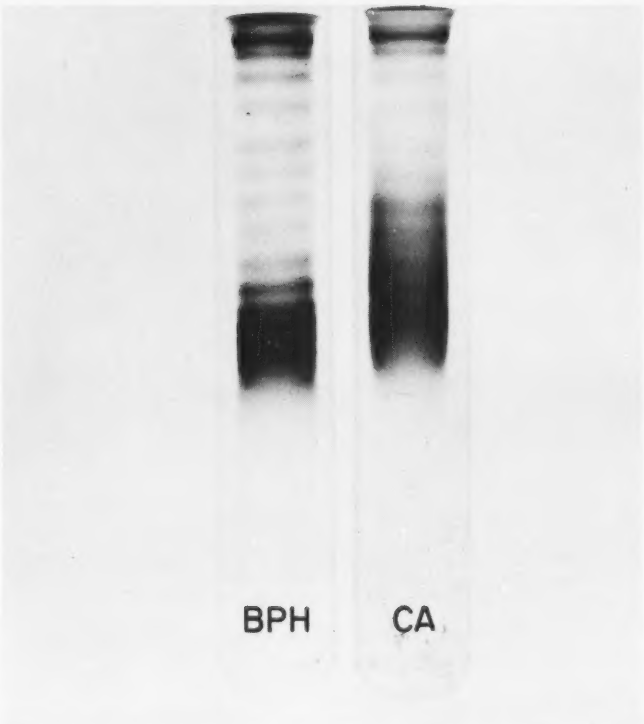

FIGURE 3.—Isoenzyme patterns of acid phosphatase in benign hypertrophic (BPH) and cancerous (CA) prostatic tissues. (*See* fig 1.)

TABLE 1.—RNase and acid phosphatase activities in patients with prostatic carcinoma

Patient No.	RNase activity*	Acid phosphatase activity†
1	220	3.4
2	255	364.0
3	260	1.9
4	260	2.3
5	267	324.5
6	270	3.6
7	275	28.4
8	295	15.2
9	300	45.2
10	310	67.5
11	315	99.7
12	320	7.6
13	320	33.8
14	333	26.3
15	335	32.4
16	335	94.4
17	340	94.4
18	355	241.3
19	360	142.9
20	370	2.2
21	390	106.2
22	390	255.7
23	415	1.8
24	435	713.8
25	440	13.2
26	460	91.8
27	725	133.1

*Normal range, 92–296.
†Normal range, 1.0–4.0.

and 78% respectively when either assay was used alone.

It has been reported that the elevation of serum RNase activity was associated with other neoplasms (3). The origin of serum RNase from patients with prostatic cancer merits further study. It is of diagnostic importance to show that this serum RNase originates from the prostatic gland, and that an assay can be developed for the specific serum prostatic RNase.

Immunologic Study of Prostatic RNase

To study whether the prostatic RNase is immunologically distinct from RNase of other tissues, RNase was purified from the prostate and antiserum to the purified enzyme was produced in rabbits. The results indicated that the antiserum reacted with both purified prostatic RNase and serum from the prostatic cancer patient which contained elevated RNase activity. To ensure that our antiserum contained no contaminating antibodies to other serum components, RNase antiserum was absorbed with NFS. As shown in figure 4, the NFS-absorbed antiserum formed one major precipitin line and one faint line against purified prostatic RNase in Ouchterlony's double diffusion (well 4 vs well 5). The NFS-treated antiserum also cross-reacted with RNase in PCA extract of ascites fluid

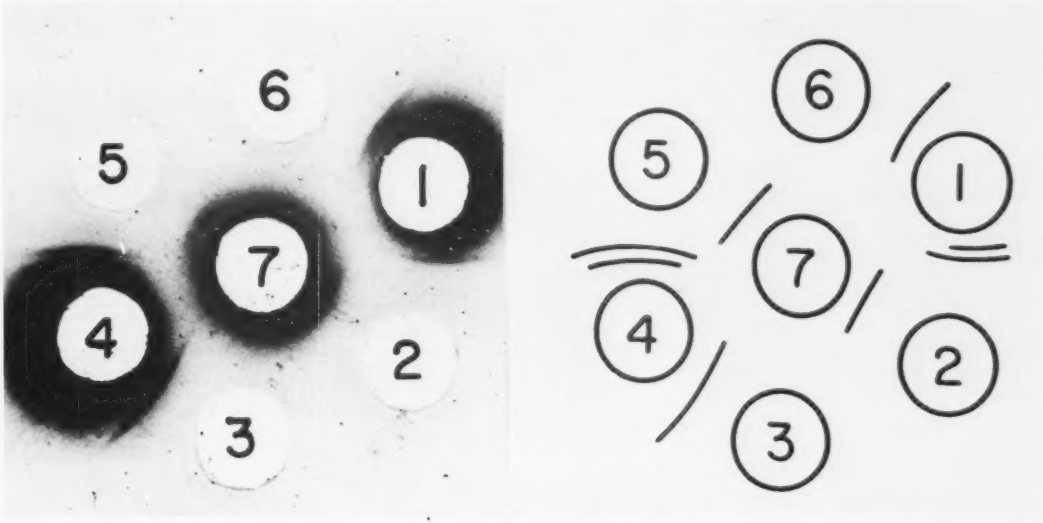

FIGURE 4.—Double immunodiffusion of rabbit antiserum to human prostatic RNase. Well 1, antiserum absorbed with NFS and bovine pancreatic RNase (1 mg/ml); wells 2 and 5, purified prostatic RNase (0.1 mg/ml); wells 3 and 6, human pancreatic RNase (0.4 mg/ml); well 4, antiserum absorbed with NFS; and well 7, antiserum absorbed with NFS and human pancreatic RNase.

obtained from a patient with pancreatic cancer, but only one precipitin line was observed (well 3 vs well 4). Further absorption of NFS-treated antiserum with the PCA extract abolished the formation of this precipitin line (well 3 vs well 7 and well 6 vs well 7). Absorption with PCA-extract of pancreatic carcinoma also abolished the major precipitin line formed between the antiserum and purified prostatic RNase, but the faint second line remained (wells 2 and 5 vs well 7). Additional experiments by absorption with bovine pancreatic RNase did not alter the precipitin lines between antiserum (well 1) and prostatic RNase (well 2) or the PCA extract of pancreatic carcinoma (well 6), indicating that human prostatic RNase and pancreatic RNase were immunologically different from the bovine enzyme. These results indicated that our rabbit antiserum produced from prostatic RNase may contain two populations of antibodies, one of which would react with pancreatic RNase. It remains to be determined whether or not these two components represent two isoenzyme forms of the purified prostatic RNase preparation. Further investigation is now in progress.

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Immunochemical Studies of Prostatic Acid Phosphatase[1]

B. K. Choe,* E. J. Pontes, I. McDonald, and N. R. Rose[2]

SUMMARY

Immunologic specificity of the prostatic acid phosphatase (PAP) and the kinetics of the PAP-anti-PAP antibody interactions were studied in detail. Antigenic identity of electrophoretic isozymes of PAP was established in this study. Counter-immunoelectrophoretic assay, radioimmunoassay, and immunofluorescence technique were developed to detect PAP and PAP-synthesizing cells. These techniques were applied to the determination of serum PAP levels and to the identification of metastatic prostate cancer cells in biopsies.

[Cancer Treat Rep 61:201–204, 1977]

Elevation of serum acid phosphatase level has long been regarded as a useful indicator in the diagnosis of metastatic carcinoma of the prostate (1,2). Although most of the present biochemical determinations lack specificity to recognize the tissue origin of the enzyme, recent evidence suggests that prostatic acid phosphatase (PAP) is antigenically distinct from acid phosphatases of other tissue origin (3). Based on this immunochemical specificity, a few immunoassay methods have been proposed for this enzyme (4–6). In this paper we have summarized our work on the immunochemical characterization of PAP and assessed the feasibility of immunoassay for this enzyme. We have also discussed the significance of immunologic studies of acid phosphatases of other tissue origins in relation to the interpretation of immunoassays for the PAP.

Immunochemical Characterization of PAP

The enzyme was purified according to the general procedure described by Ostrowski et al (7) with some modifications (fig 1). Each purification step was monitored by polyacrylamid gel electrophoresis and by immunodiffusion employing various reagent antisera. These test antisera included anti-PAP (immunized against crude preparation), anti-PAP (highly purified PAP isozymes as the immunogen), antisera against whole human serum, albumin, gamma globulin, IgA, IgG, IgM, transferrin, haptoglobin, and serum α and β globulin fractions. The use of affinity chromatography on concanavalin A (Con A) and immunoadsorbent column prepared by anti-whole human serum was particularly effective in removing nonglycoproteins such as albumin and trace amounts of serum proteins from the partially purified PAP preparations. Two electrophoretic or chromatographic isozymic forms of PAP were separated by ion-exchange column chromatography, which were defined as DI and DII. It has been established by previous investigators that isozymic forms of PAP arise from their charge group hetero-

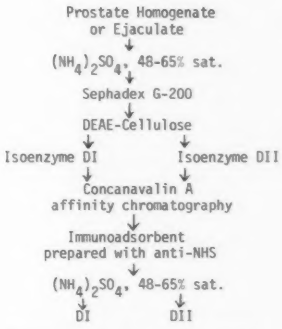

FIGURE 1.—Purification of PAP.

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geneity and that the charge heterogeneity originates from the neuraminic acid residues (7,8). We asked if the neuraminic acid residues play any significant role(s) in determining their antigenic specificity of the molecule. If so, each isozymic form may exhibit slight difference(s) in their antigenic determinants. It was particularly interesting to study this point because two research groups previously reported the occurrence of two molecular forms of PAP possibly differing in their antigenic determinants (3,9).

Quantitative precipitin reactions between rabbit anti-PAP and DI or DII showed the same antibody/antigen mol ratio of 8 at extreme antibody excess. The limiting mol ratio of antibody to antigen in antibody excess is often taken as a measure of the antigen molecule's valence, therefore the results seem to suggest that antigenic determinants on DI and DII are very similar. This notion was strengthened by the competitive inhibition of DI binding to anti-PAP antibody by DII. When ^{125}I-DI-anti-PAP antibody interactions were measured in the presence of unlabeled DII, 20 ng of DI inhibited 50% of the DI-anti-PAP interactions. When unlabeled DI was used as the competitive inhibitor of ^{125}I-DII-anti-PAP interactions, 20 ng of DI was required for 50% competitive inhibition. This type of experiment also suggested the antigenic identity of DI and DII.

To verify this presumptive evidence further, we have used neuraminidase-digested DI and DII. Neuraminidase digestion of isozymes did not reduce their enzyme activity greatly. However, the digested isozymes DI and DII showed a very similar isoelectric point, pH 5.7–6.0. The antigenic identity of undigested and digested DI and DII was examined by immunodiffusion to anti-PAP serum. The line of identity was observed between DI and DII with or without neuraminidase digestion.

The PAP is a glycoprotein of approximately 100,000 daltons. A slight molecular heterogeneity observed by a few investigators may be related to the variation of carbohydrate contents of isozymic forms. We think that the PAP isozymes are antigenically identical and the carbohydrate residues of the PAP molecules are insignificant in determining the antigenicity of this molecule. Further experimental results are summarized in table 1.

Specificity of Anti-PAP Antibodies

Anti-PAP antibodies were raised in monkeys and rabbits and the quantitative aspects of PAP-anti-PAP antibody interactions were studied in detail. Most of our anti-PAP sera belong to the class of avid antiserum exhibiting relative affinity of 2.2×10^7–1.2×10^8 liters/mol.

Specificity of anti-PAP sera was examined by serial absorption with normal human sera-immunoadsorbent and by testing it against PAP and various acid phosphatase preparations. Acid phosphatases were prepared from a number of tissues and cell cultures which included pancreas, liver, lung, lymphocytes, breast carcinoma cells (MCF-7), uterine cancer cells (HeLa), fibroblasts (WI-38), and putative prostatic epithelial cells (MA-160 and EB-33). When anti-PAP sera were tested against acid phosphatases of nonprostatic origin, none of them was recognized by anti-PAP sera. We have confirmed immunologic specificity of PAP suggested by previous investigators (3). However, it should be noted here that the bulk of the pancreatic acid phosphatase did not react with anti-PAP antibody,

TABLE 1.—Characterization of PAP isoenzymes

Purification	Isozyme DI (57–80-fold)	Isozyme DII (28–56-fold)
Specific enzyme activity (units/mg protein)	1965	960–1200
Immunochemical homogeneity	No extraneous proteins	Contains extraneous proteins
Approximate molecular weight	100,000	100,000
Approximate pI	4.8	4.5
K_m	6.2×10^{-4} M	5.6×10^{-4} M
V_{max}	0.04 μmol/min	0.20 μmol/min
Con A binding	Yes	Yes
Immunochemical identity	Nearly identical with DII	Nearly identical with DI
Antigenic determinants	4–8	4–8

TABLE 2.—Serum PAP levels in normal men and cancer patients

Patients	RIA		CIEP	
	No. of cases*	ng of PAP/100 μl	No. of cases	ng of PAP/100 μl
Normal men (age, 23–40 yrs)	162 (s)	1.6 ± 0.8	40 (s)	†
Benign prostatic hypertrophy	11 (s)			
	6 (B)	0.5–4.0	10 (s)	†
Nonprostatic carcinoma	10 (s)	1.8 ± 0.6	10 (s)	†
Prostate carcinoma	22 (s)	1.8–2.5	20	†
Localized	8 (B)	0.5–2.1	3	†
Metastatic	21 (s)	5.5–100.0	21	5.0–100.0
	10 (B)	8.5–180.0	10	5.0–100.0

*s = serum, B = bone marrow.
†Concentrations of PAP < 2.5 ng/100 μl are not measurable by CIEP assay.

but a small fraction of it (possibly < 5% of the total) was cross-reactive with PAP in the immunoelectrophoresis and by radioimmunoassay (RIA). Experiments are in progress to settle the question of whether this minor cross-reactive enzyme is synthesized by a special cell type in the pancreas or by the whole pancreatic tissue. Therefore, it became increasingly evident that immunochemical studies should be extended to acid phosphatases of other tissue origin in order to define the antigenic specificity of the PAP.

Preliminary Evaluation of PAP Immunoassays

A double-antibody RIA and counterimmunoelectrophoretic (CIEP) assay were developed for the determination of serum PAP levels, and an indirect immunofluorescent antibody (IF) technique was also developed for the identification of PAP-secreting cells. The protocols of these immunoassays employed in our laboratory are summarized in figure 2. Serum PAP levels were determined by RIA and CIEP in 165 normal men and in 48 prostate carcinoma patients and the results are summarized in table 2.

The normal serum PAP level was found to be 1.6 ± 0.8 ng/100 μl. The serum PAP levels of ten patients with malignant but nonprostate tumors fell in the normal range, whereas levels > 4 ng/100 μl were found in patients with prostate carcinoma. Serum PAP levels determined by CIEP showed close agreement with those obtained by RIA for the same patients. The number of cases is too small to draw any conclusions as yet. However, it seems clear that immunoassays have specificity and higher sensitivity than activity assays of this enzyme. It may be noteworthy to mention that the minimum level of PAP concentration detectable by CIEP is 2-3 ng/100 μl, therefore, the PAP levels detectable by this method seem to represent a borderline case of serum PAP elevation. This technique is quick, economic, and simple enough to be used at the clinical laboratories.

A. CIEP: (a) 10 μl samples to cathodal wells and 5 μl anti-PAP serum to anodal wells are electrophoresed for 45 min. PAP-anti-PAP complexes are stained histochemically.
(b) Serially diluted standard PAP gives an estimate of PAP concentrations.

B. RIA: (a) 100 μl of 1:2,000 dilution of anti-PAP + 5-10 ng ^{125}I-PAP, + sample (usually 100 μl) → incubate at 4°C for 24 hr.
(b) 5 μl goat anti-rabbit IgG is added → additional 24 hr at 4°C.
(c) Binding of ^{125}I-PAP in sample tubes are determined and compared with standard binding curve.

C. IF: (a) Diluted anti-PAP serum → frozen section or paraffin section of tissues for 30 min at room temp., then washed.
(b) Chicken anti-rabbit IgG globulins conjugated with FITC → anti-PAP reacted slides for 30 min at room temp., then washed.
(c) UV microscopy.

FIGURE 2.—Immunoassay protocols for PAP.

The aim of the IF technique for PAP-secreting cells is to be applied to the detection of presumptive prostatic carcinoma cells metastasized to the bone marrow and/or lymph nodes. For the evaluation of the specificity of this technique, various tissue specimens were examined and various types of reconstruction experiments were performed (10). This IF method is specific for the detection of PAP-secreting cells. A few cases of metastatic prostatic carcinoma showed IF-positive cells in their bone marrow biopsies. However, reconstruction experiments with EB-33 and MA-160 cells indicate that there are large numbers of IF-negative cells among PAP-secreting cells. This suggested a few possibilities, ie, (*a*) the PAP synthesis may be limited to a certain cell-cycle period, (*b*) the PAP synthesis is regulated by hormones, and (*c*) the PAP becomes IF positive only when it is packaged at the Golgi apparatus into secretory granules.

Significance of Immunochemical Studies

The acid phosphatases are a lysosomal enzyme in most cells. However, most of the acid phosphatase seems to be secretory enzyme in prostatic epithelial cells which may function as a generator of phosphate and choline in the semen. Since the enzyme is found both in lysosomes and in secretions of one type of cell population, a general question to be asked is: are the lysosomal enzyme and the secretory forms transcribed from the same or different gene(s)? This question has never been properly answered.

The PAP seems antigenically unique in the sense that anti-PAP antibodies do not react with acid phosphatases originated from other tissues. Does this mean that genetic loci determining acid phosphatase synthesis are multiple in human cells and each tissue transcribes different acid phosphatase genes? Two years ago, we observed a case of pancreatic islet cell carcinoma metastasized to the liver, in which the tumor produced acid phosphatase which was immunologically cross-reactive with the PAP (unpublished observation). In this case, the possibility of prostatic involvement was convincingly ruled out by the clinical study and by the postmortem pathologic studies. As mentioned in the section on Specificity of Anti-PAP Antibodies, bulk pancreatic cell acid phosphatase does not share antigenic specificity with PAP. However, a small fraction of pancreatic acid phosphatase is immunologically cross-reactive with PAP. Did the tumor originate from a clone of pancreatic cell subpopulation which secretes the cross-reactive acid phosphatase? Work is still in progress to examine this question in our laboratory. This curious observation raised many fundamental questions concerning the immunochemical classification of acid phosphatases and molecular mechanisms of acid phosphatase polymorphism. We could not feel confident in the interpretation of PAP immunoassays until most of these questions are answered.

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Analysis of Prostatic Fluid in Prostatic Disease[1]

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SUMMARY

The current studies of expressed prostatic fluid tend to confirm our previous reported pilot observations of a shift in lactic dehydrogenase (LDH) isoenzymes to a predominance of LDHV in patients with prostatic malignancy. They also suggest a decrease in concentration of acid phosphatase and an increase in concentration of protein in the presence of carcinoma of the prostate. These observations suggest a diffuse metabolic alteration of the prostate in the presence of prostatic carcinoma.

[Cancer Treat Rep 61:205–210, 1977]

This study of enzymes and other biochemical constituents of expressed prostatic fluid was undertaken to attempt to identify men with a high probability of the existence of early carcinoma of the prostate or a high probability of developing this lesion. The observation of a diffuse change characteristic of malignancy (1) in the isoenzyme pattern of lactic dehydrogenase (LDH) in prostates with limited histologic evidence of malignancy by Oliver et al (2) indicated a widespread metabolic alteration preceding or coincident with the development of recognizable malignancy. The predominance of the LDHV isoenzyme in histologically normal cells apparently maintaining a normal acinar relationship coupled with the knowledge that prostatic fluid represents an apocrine or merocrine secretion suggested that prostatic fluid would reflect the metabolic alterations present. These assumptions and preliminary observations of LDH isoenzymes (3) have led us to continue to evaluate LDH isoenzymes and to initiate studies of acid phosphatase and protein concentration as well as other constituents of prostatic fluid in men with clinically normal and clinically or histologically established prostatic pathology. This is a report of the current status of this effort.

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METHODS

Prostatic fluid obtained by digital massage 10–30 minutes after voiding is usually collected on a glass slide. After transfer to a small plastic or glass container, the fluid is placed in a refrigerator at 4°C and analyzed promptly or frozen for later analysis. A cover slip is placed on the residual fluid on the slide and microscopic evaluation of the cells and particles present is carried out promptly. When quantities of fluid permit, duplicate and sequential evaluation of cooled and frozen samples is carried out. To date, 876 prostatic fluid samples from 628 patients have been subjected to one or more of the analyses detailed below. Histologic diagnosis of the prostatic pathology present has been determined on tissue removed at transurethral or enucleation prostatectomy or at needle biopsy in 106 of these patients.

The expressed prostatic fluid is subjected to LDH isoenzyme, acid phosphatase, leucine amino peptidase, glucose-6-phosphate dehydrogenase, and protein determination. All prostatic fluid specimens are subjected to LDH isoenzyme determination. When possible, the other determinations are carried out. Only LDH isoenzyme, acid phosphatase, and protein concentrations are considered in this report.

LDH isoenzymes are determined by a slight modification of the method described by Dietz and Lumbrano (4). Five μl of prostatic fluid is layered on top of 5.5% of acrilamide gel; 40 μl of 40% sucrose solution is added followed by a further gel layer. After 45–60 minutes electrophoresis is carried out for 45 minutes at room temperature. After rinsing, the gels are stained with modified van der Helm

reagents and placed in an incubator for 30–60 minutes at 37°C before reading in a Photovolt Densicord densitometer.

Prostatic fluid acid phosphatase is determined on fluid diluted 1:7500 with distilled water using sodium thymolphtholein substrate (5). Protein concentration was determined initially by observing the absorption at 280 mμ of prostatic fluid diluted 1:750 with distilled water. When the values obtained by this technique failed to correspond to the protein concentration determined by the Lowry et al method (6), the procedure was modified by subjecting the diluted fluid to centrifugation at 5000 rpm in a refrigerated centrifuge. Absorption at 280 mμ is then determined after the sample returns to room temperature. Bovine serum albumin is used as a standard.

RESULTS

LDH isoenzyme.—The mean LDHV/I isoenzyme ratio in prostatic fluid increases progressively with age (table 1). The mean ratio also increases when

TABLE 1.—Mean LDH isoenzyme ratios related to age

Age	No. of patients	LDHV/LDHI ± SE
< 40	142	0.834 ± 0.117
40–49	91	1.289 ± 0.217
50–59	195	1.660 ± 0.179
60–69	249	2.021 ± 0.215
≥ 70	199	2.187 ± 0.230

> 5 and > 50 wbcs are seen on high-power microscopic examination of the fluid (table 2). The ratio of all 31 determinations of the 21 patients with a histologic diagnosis of carcinoma had a mean value of 3.28 compared to the mean value of 1.68 for all 328 determinations on the 231 patients who had a histologic or clinical diagnosis of benign prostatic hyperplasia (BPH) (table 3). Repeated sampling at short intervals of prostatic fluid for enzyme determination seems to be associated with a tendency to decrease the LDHV/I ratio. Figure 1 is a graph of the highest LDHV/I ratio in men with histologic diagnoses of carcinoma of the prostate and BPH.

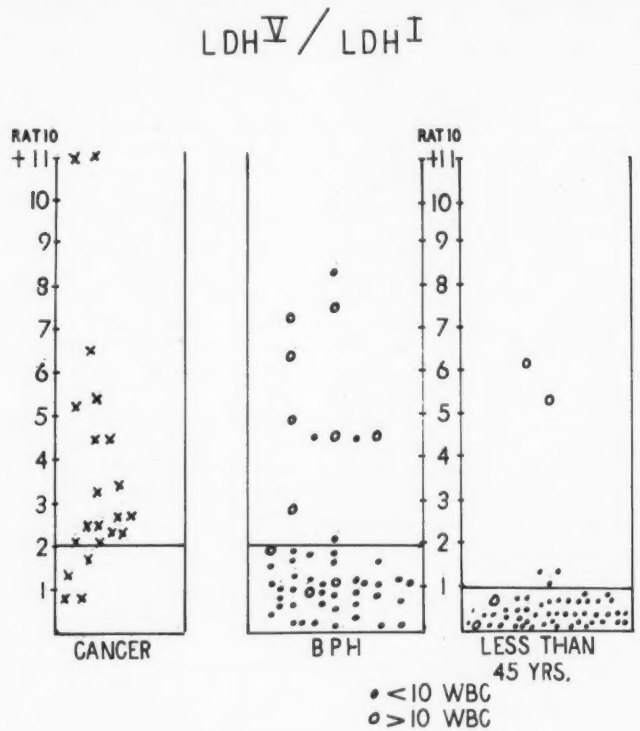

FIGURE 1.—Maximum LDHV/I ratio observed in prostatic fluid of patients with histologic diagnosis of carcinoma and BPH and in men < 45 years of age.

TABLE 2.—Mean LDH isoenzyme ratio related to wbcs per hpf*

Group	No. of patients	LDHV/LDHI ± SE
< 5 wbc/hpf	596	1.318 ± 0.085
5–50 wbc/hpf	180	2.345 ± 0.192
≥ 50 wbc/hpf	31	4.984 ± 1.265

*Correlation coefficient = 0.289, N = 807, $P < 0.001$.

The observed ratio in 54 men < 45 years of age in whom a diagnosis of either carcinoma or BPH is unlikely is also presented. The LDHV/I ratio is > 2 in 17 of 21 patients with carcinoma and is < 2 in 37 of 41 men with a histologic diagnosis of BPH who do not have evidence of inflammation. Forty-seven of 50 men < 45 years of age who had < 10 wbcs/high-power field (hpf) on microscopic examination have a ratio of < 1 and three men have a ratio of < 1.5.

TABLE 3.—Mean of all LDH isoenzyme ratio determinations in patients with a histologic diagnosis of cancer, histologic and clinical diagnosisof BPH, prostatitisas judged clinically, and normal prostates on clinical evaluation

Group	No. of patients	LDHV/LDHI ± SE
Cancer	31	3.280 ± 0.705
BPH	328	1.683 ± 0.171*
Prostatitis	183	1.986 ± 0.192
BPH and prostatitis	49	1.949 ± 0.236
Normal†	37	0.484 ± 0.093‡

*Significantly different from cancer, $P < 0.05$.
†Patients with no genitourinary diseases.
‡Significantly different from cancer, $P < 0.001$.

Acid phosphatase.—The acid phosphatase/g of protein equivalent as determined by ultraviolet (UV) absorption at 280mμ has been determined in 14 patients with a histologic diagnosis of carcinoma, 43

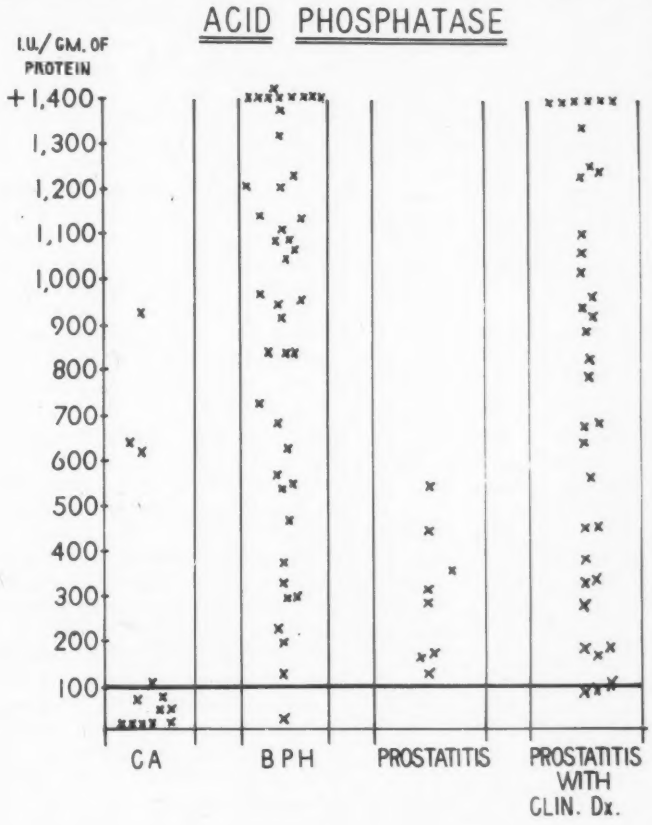

FIGURE 2.—Acid phosphatase per g of protein equivalent in patients with histologic diagnosis of cancer, BPH, and prostatitis, and in patients with ≥ 10 wbcs/hpf on microscopic examination of prostatic fluid.

patients with a histologic diagnosis of BPH, and 59 patients with ≥ 10 wbcs/hpf in the prostatic fluid (table 4). The mean acid phosphatase concentration was 241.6 ± 9.1 in the carcinoma group, 1106.4 ± 143.4 in the BPH group, and 1351.5 ± 190.4 in the patients with evidence of prostatic inflammation. Individual values for patients in these groups are shown in figure 2; ten of 14 patients with carcinoma had an acid phosphatase level < 100 units/g of protein equivalent. Only one of 43 patients with BPH and two of 59 with prostatic inflammation were below this level.

Protein concentration.—The limited amount of prostatic fluid available for multiple analyses led to the determination of protein concentration as a reference for enzyme content. Initially, the standard UV absorption at 280 mμ was utilized on diluted fluid and presumed to reflect its protein content with reasonable accuracy. A disparity in results obtained with this technique and the Lowry method became evident when large volume samples were analyzed by both procedures. As a result, the assumption of validity of the absorption procedure was evaluated critically and correspondence between the two methods was achieved by centrifugation of the diluted fluid. Limited observations on uncentrifuged and centrifuged fluid are available at this time; both groups seem to indicate a definite increase in protein content of the prostatic fluid from patients with prostatic malignancy compared to those with BPH (table 5).

TABLE 4.—Mean acid phosphatase concentration per g of protein equivalent determined by absorption of uncentrifuged prostatic fluid at 280 mμ in patients with histologic diagnosis of cancer, BPH, and clinical diagnosis of prostatitis

Group	No. of patients	IU/g of protein (mean ± SE)
Cancer	14	241.64 ± 9.11
BPH	43	1106.44 ± 143.40*
Prostatitis	59	1351.50 ± 190.37*

*Significantly different from cancer, $P < 0.001$.

TABLE 5.—Mean protein concentration determined by absorption of centrifuged prostatic fluid at 280 mμ in patients with histologic diagnosis of BPH or cancer

Group	No. of patients	mg/ml (mean ± SE)
BPH	18	84.78 ± 8.23
BPH and prostatitis	3	56.50 ± 7.37
Cancer	3	138.75 ± 17.73*

*$P < 0.05$ between cancer group and others.

DISCUSSION

These observations support the presence of identifiable biochemical abnormalities in the expressed prostatic fluid of men with carcinoma of the prostate. The changes in LDH isoenzymes, acid phosphatase, and protein concentrations provide further evidence of a diffuse cellular change in the prostate associated with the presence of a prostatic malignancy. The current study confirms and extends the finding previously reported by our group (3) of a relative increase in the LDHV isoenzyme in the presence of carcinoma compared to BPH. The apparent increase in the ratio of the V/I isoenzyme again seems to be unrelated to the grade or stage of the malignancy. The suggestion that inflammation of the prostate as reflected by an increased number of wbcs in prostatic fluid was associated with an increased V/I isoenzyme ratio was also confirmed. Preliminary observations indicated that the isoenzyme ratios do not always return to a predominance of lighter isoenzymes promptly with reduction of wbc counts in the fluid to normal levels. Confirmation of this impression and evaluation of its significance will hopefully be possible with continuation of the study.

Assessment of the limited number of determinations of acid phosphatase and protein concentration in prostatic fluid also supports the suggestion of a difference between patients with carcinoma and BPH. These data with regard to acid phosphatase concentration must be accepted with some reservation for two reasons relating to the use of protein concentration as a reference standard. First, the uncentrifuged diluted prostatic fluid was used for UV absorption studies of most of the prostatic fluid specimens reported; although the protein concentration estimated by this technique is abnormally high, the procedure does yield information readily reproduced by other groups. Retention of the data with designation of the reference unit as "protein equivalent" seemed justified. Second, the assumption of a relatively constant protein concentration despite a disease state does not seem to have been warranted. An apparent increase in protein concentration in the prostatic fluid of men with carcinoma would result in a decreased concentration of acid phosphatase on a protein weight basis even if the concentration was maintained on a volume basis. We have chosen to include the acid phosphatase data because they are reproducible, current observations utilizing centrifuged diluted fluid for protein determination seem to be confirming these data, and the data are supported by the observations of Kent et al (7) of acid phosphatase concentration in patients with stage 3 and 4 prostatic malignancy. Since our obser-

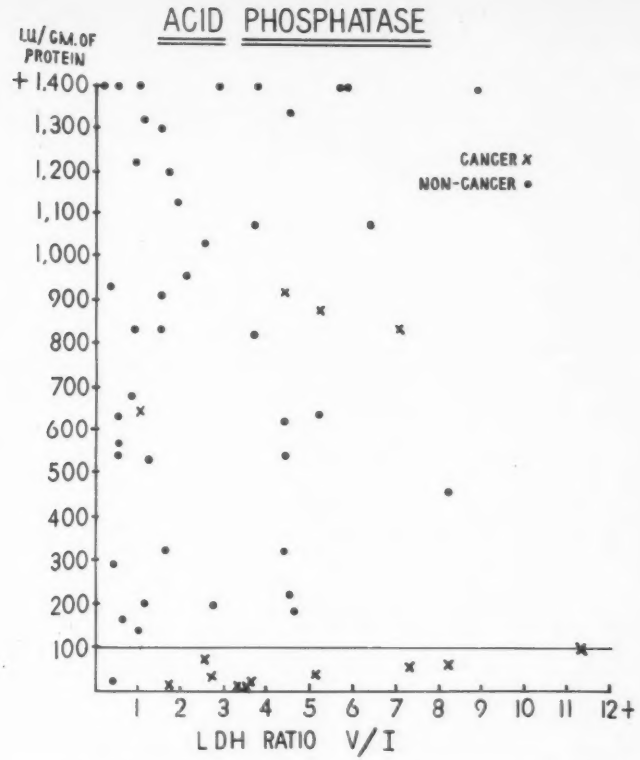

FIGURE 3.—Relationship of LDHV/I ratio and acid phosphatase per g of protein equivalent in prostatic fluid of patients with a histologic diagnosis of cancer and BPH.

vations have been made in patients with all stages of malignancy and since we have experienced difficulty in obtaining fluid from patients with a diffuse local malignancy, the assumption that the prostatic fluid changes noted reflect a diffuse cellular change in the prostate rather than a sampling of the histologically malignant cell mass seems reasonable.

The preliminary observations utilizing centrifuged diluted prostatic fluid seem to indicate an increased protein concentration in patients with carcinoma of the prostate. If this surprising change is confirmed by further study, then the qualitative nature of the protein and identification of its source require investigation.

Figure 3 is an attempt to correlate changes in acid phosphatase concentration per g of protein equivalent and LDHV/I ratios in the fluid of patients with proven carcinoma of the prostate and histologically diagnosed BPH. The LDH isoenzyme ratios and acid phosphatase concentration both seem to be altered in most patients with malignancy. However, there are some patients with an acid phosphatase concentration < 100 units/g of protein equivalent and an LDHV/I ratio < 2; others have an LDHV/I ratio > 2 and an acid phosphatase concentration in the normal range. These observations suggest the possibility of isolated metabolic alterations that may identify malignancies with different biologic potential.

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Tumor-Specific Immunity in Patients With Prostatic Adenocarcinoma or Benign Prostatic Hyperplasia [1]

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SUMMARY

Twenty patients were skin tested with solubilized components of autologous prostatic adenocarcinoma or benign prostatic hypertrophy (BPH). Four of ten patients bearing adenocarcinoma of the prostate exhibited tumor-specific cutaneous responsiveness to solubilized autologous tumor-specific antigens (TSA). One responding patient also responded to solubilized allogeneic TSA. A single patient bearing prostatic carcinoma responded only to solubilized components of allogeneic BPH. Nine of ten patients with BPH exhibited no response to solubilized components of autologous BPH. The patient responding to the extract of autologous BPH, however, also had clinical stage B adenocarcinoma of the prostate. These observations suggest host responsiveness to TSA of prostatic carcinoma. Possible clinical significance and mechanisms to explain the findings are discussed.

[Cancer Treat Rep 61:211–216, 1977]

The original tumor transplantation studies of Gross (1), Foley (2), and Prehn et al (3) have been succeeded by convincing data demonstrating that most solid tumors bear tumor-specific antigens (TSA). By action on TSA, the host's immune system may influence tumor progression. On the other hand, studies using prostatic carcinoma in vitro have not provided definitive evidence for the existence of TSA. However, suspicions for influences of patients' immune systems on prostatic tumor progression are raised by indirect evidence: increased incidence of prostatic carcinoma and of immunologic defects with advancing age (4), the state of latency (5), the prolonged natural course of some patients (5–7), and the presence of TSA in most other solid tumors. Experimental evidence for such host-tumor immunorelationships includes alterations in lymphocyte blast-transformation (8–11), depressed T-lymphocyte levels (12), and decreased cell-mediated immunocompetence (13) in prostatic cancer patients. The demonstrations of elevated carcinoembryonic antigen (14–16) and the rare reports of regression of metastases following cryosurgery to the malignant prostate (17–20) may also suggest host immune influence.

The demonstrations of cutaneous responsiveness to solubilized autologous TSA in patients with Burkitt's lymphoma (21,22), malignant melanoma (23–25), leukemia (26–30), and carcinoma of the cervix (31,32), colon (33,34), breast (35,36), lung (31,37,38), and others (39,40) substantiated our interest in eliciting cutaneous responses to intradermal injections of prostatic tumor antigens solubilized using hypertonic (3 M) potassium chloride. The purpose of the present study was to determine the specificity of cutaneous reactivity to solubilized autologous tumor antigens in patients bearing adenocarcinoma of the prostate and benign prostatic hypertrophy (BPH).

MATERIALS AND METHODS

Neoplastic or benign hyperplastic prostatic tissue was obtained from patients by means of transurethral resection, using the cutting current and the Stern-McCarthy Resectoscope. The tissue was minced in Hanks' balanced salt solution and washed by centrifugation. Solubilization of TSA was carried

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out using hypertonic (3 M) potassium chloride, a modification of the procedures described by Meltzer et al (41). Minced tumor tissue was suspended in 10 ml of 3 M potassium chloride in 0.005 M potassium phosphate buffer (pH 7.4) per g of wet weight of the initial tumor tissue. This suspension was kept at 4°C and intermittently agitated for 16–24 hours. All subsequent steps for extraction were performed at 4°C. After the initial extraction, the suspension was centrifuged at 40,000 × *g* for 60 minutes. The supernatant was dialyzed against 200 volumes of deionized water for 16–24 hours, the dialysis fluid being changed three times. Centrifugation of the dialysate at 40,000 × *g* for 15 minutes removed the gelatinous precipitate. The supernatants were passed directly through a 0.25μ Millipore filter into a sterile flask. They were frozen to –80°C in acetone and dry ice and lyophilized. When lyophilization was complete, the powder was reconstituted in sterile saline to a protein concentration of 7.5 mg/ml. Bacterial cultures of the extracts were negative prior to skin testing.

In addition, the cell membranes of whole blood were extracted to soluble form by the same method of hypertonic potassium chloride. The solubilized components of blood served as control tissue and were compared to solubilized components of prostatic tissue taken from the same patient.

The average age of the patients with malignant disease was 66.3 years, while the average age of patients with BPH was 62.0 years. Patients were skin tested between 4 and 6 days following transurethral resection. Patients bearing adenocarcinoma of the prostate had not been treated with X-irradiation or endocrine therapy prior to skin testing. The stage of malignant disease was determined by rectal palpation, serum acid phosphatase, metastatic survey, technetium-99m bone scan, and in some instances pelvic lymphadenectomy.

Cutaneous responses were tested by administering 0.10 ml of experimental or control solubilized antigens intradermally in the forearm. The sites of injection were inspected 24 and 48 hours after antigen administration for cutaneous reactivity. An area of at least 5 × 5 mm of palpable induration at both 24 and 48 hours was considered a positive delayed type hypersensitivity cutaneous response. Negative responses had no induration. In all cases where a positive response occurred, histologic interpretation of skin biopsies was consistent with classic criteria for delayed type hypersensitivity. Biopsies of nonresponding sites of control tissue injection revealed an absence of cellular infiltrate.

All patients were skin tested with extracts from autologous prostatic tissue (adenocarcinoma or BPH), autologous blood cells, and allogeneic hyperplastic prostate. Tissue controls included extracts of blood taken from allogeneic tissue donors. All solubilized prostatic tissue antigens were tested in age-matched control patients. Under these circumstances no cutaneous responses were observed. In some instances, patients bearing adenocarcinoma of the prostate and not showing a cutaneous response to TSA were tested for immunocompetence by intradermal skin tests using 0.10 ml of six commonly encountered antigens. These antigens included intermediate-strength purified protein derivative, 0.1 μg or 5 tuberculin units/0.10 ml; mumps skin antigen; varidase (streptokinase-streptodornase mixture), 40 and 10 units respectively; candida albicans extract, 1:100 dilution of full-strength antigen; Trichophyton gypsum extract, 1:30 dilution; and histoplasmin, 1:100 dilution. Patients with reactive immune systems generally have a delayed type hypersensitivity cutaneous response to at least two of these six antigens. Patients responding to none of these antigens are considered anergic.

RESULTS

Ten patients bearing adenocarcinoma of the prostate, clinical stage C or D, and ten patients with BPH were tested with solubilized components of autologous tumor (carcinoma or BPH) and autologous blood. In addition, all patients were skin tested using extracts of allogeneic BPH and control extracts of blood cells from the allogeneic tissue donors. The results of cutaneous responses in patients bearing adenocarcinoma of the prostate are summarized in table 1. Four of the ten patients showed a delayed type hypersensitivity cutaneous response specific to the tumor antigens extracted from autologous neoplasm. In one instance (patient 3) a mild cutaneous response was also observed to allogeneic TSA. A single patient (patient 10) surprisingly showed mild responsiveness to solubilized components of allogeneic BPH in the absence of responsiveness to autologous or allogeneic solubilized TSA. In no instances did solubilized components of blood cells elicit a cutaneous response. Histologic studies of skin biopsies from the responding sites were consistent with classic criteria for delayed type hypersensitivity. Three of the five patients not responding to solubilized autologous tumor antigens were available for further testing. All were anergic, as determined by lack of cutaneous responses to six commonly encountered antigens previously mentioned.

The results of cutaneous responses in patients with BPH are presented in table 2. Only a single patient exhibited cutaneous responsiveness specific to autologous solubilized tumor antigen. In this

TABLE 1.—Skin test responses to solubilized antigens from adenocarcinoma of the prostate*

Patient No.	Stage of prostatic adenocarcinoma	Cutaneous responses: mm² of induration in response to— Autologous extract of prostatic carcinoma	Allogeneic extract of prostatic carcinoma	Allogeneic extract of BPH	Tumor-specific response: Autologous	Tumor-specific response: Allogeneic	Anergy
1	D	8 × 8	0	0	+	0	
2	D	6 × 6	0	0	+	0	
3	C	7 × 7	6 × 7	0	+	+	
4	C	5 × 6	0	0	+	–	
5	D	0	0	0	–	–	Yes
6	C	0	0	0	–	–	?
7	D	0	0	0	–	–	?
8	D	0	0	0	–	–	Yes
9	D	0	0	0	–	–	Yes
10	D	0	0	5 × 5	–	+ (BPH)	

*No cutaneous responses were elicited by injection of solubilized components of autologous or allogeneic blood.

TABLE 2.—Skin test responses to solubilized antigens from BPH*

Patient No.	Autologous extract of hyperplastic prostate	Allogeneic extract of hyperplastic prostate
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10†	5 × 6	0

*No cutaneous responses were elicited by injection of solubilized components of autologous or allogeneic blood.

†Original diagnosis was stage B adenocarcinoma of the prostate (confirmed by needle biopsy). Tissue obtained by transurethral resection in this elderly patient was exclusively BPH. Solubilized components of the benign tissue elicited the cutaneous reactivity noted.

unusual instance stage B-1 adenocarcinoma was suspected on digital examination and confirmed by needle biopsy. The tissue obtained by transurethral resection was exclusively BPH in the portion examined. Cutaneous responsiveness to solubilized components of the tissue obtained could represent a response to solubilized prostate carcinoma not identified or a response by the patient sensitized to antigens common to both carcinoma and BPH.

DISCUSSION

The observation that four of ten patients bearing adenocarcinoma of the prostate exhibited cutaneous delayed hypersensitivity responses specific to solubilized autologous TSA suggests that adenocarcinoma of the prostate may bear TSA to which the host may respond. This 40% cutaneous responsiveness compares favorably with similar studies using other solid tumors. Although Hughs and Lytton (40) reported 27% of 33 patients responding to autologous tumor extracts, two patients reacted both to solubilized control and to solubilized tumor tissue. Stewart (39) found that 26% of 134 solid tumor patients responded to autologous tumor preparations; however, six patients responded to autologous control skin preparations as well. In lung carcinoma (31,38) 50% of 14 patients responded to autologous tumor membrane extracts, while two of the seven responders also reacted to autologous leukocyte extracts. Four of six patients with carcinoma of the cervix responded to extracts of autologous tumor; one patient responded to solubilized components of autologous leukocytes (31). Specificity and sensitivity of tumor-specific cutaneous responsiveness has been high in studies with carcinoma of the breast and colon. Forty-six percent of 13 patients with carcinoma of the breast (35,36) and 90% of 19 patients with carcinoma of the colon and rectum (33,34) showed positive responses to autologous tumor extracts and negative responses to solubilized autologous control tissues. The very high sensitivity using solubilized components of colon carcinoma probably resulted from use of a specific area eluted from Sephadex G-200 fractionation. Our results most closely resemble those found in carcinoma of the breast. Although previous studies suggest that cutaneous injections of > 300 μg of protein often result in nonspecific skin responses, the protein concentration of our solubilized components was more than twice the threshold dose of 300 μg and more than fourfold the concentration used in breast

studies. Although the "nonspecific responses" (observed at high protein concentrations and in some patients with solid tumors) could represent an autoimmune reaction to control leukocytes, this phenomenon has not been observed in patients with carcinoma of the breast or prostate. A more likely explanation for the increased specificity and sensitivity in our studies would be that the present method permits more selective solubilization of components of TSA (41–45). The increase in sensitivity and specificity of this new method of solubilization (even in the absence of further purification procedures by precipitation in 4 M ammonium sulfate or by fractionation on Sephadex G-200 column) may account for the large percent of positive responses and lack of nonspecific responses in the present series. In addition, the use of whole blood as control tissue may have led to solubilization of fewer components of cell-mediated immunity normally present in the buffy coat.

One of the four responding prostatic carcinoma patients exhibited a cutaneous response to allogeneic tumor extracts. The findings in this patient may represent a response to TSA common to autologous and to allogeneic prostatic carcinoma. Patients with leukemia, while in remission, show cutaneous reactivity to antigen preparations of allogeneic blast cells. As in the present study, the incidence of reactivity to autologous preparations is greater than to allogeneic preparations (46). These data are consistent with the existence of both individually specific antigens plus cross-reacting antigens, common to leukemic blast cells from more than one person (46). Wells et al (38) noted cutaneous reactivity in two patients with lung carcinoma to allogeneic solubilized antigens from tumors of histologic type similar to that of the patient tested. Eight of 18 patients with carcinoma of the cervix reacted to extracts from allogeneic tumor of similar histologic type (31). Initial efforts to isolate antigens common to allogeneic tumors have been successful. A specific Sephadex G-200 fraction of solubilized components of malignant melanoma produced a delayed hypersensitivity reaction in 12 of 15 allogeneic tests. Antigens solubilized from carcinoma of the colon and from digestive tract cells of fetuses were isolated by elution of Sephadex fractions followed by polyacrylamide gel electrophoresis. These fractions resulted in cutaneous responses in allogeneic patients, demonstrating common antigenicity between allogeneic colon carcinomas (33,34). Finally, three of four mammary cancer patients exhibited delayed hypersensitivity to allogeneic mammary cancer membranes, but no patients responded to allogeneic benign breast membranes derived from the same breast (36). The data reviewed are consistent with the existence of both individual TSA plus cross-reacting antigens common to allogeneic tumors of similar histologic type. However, the incidence of allogeneic reactivity in the present study was lower than that found in studies of other solid tumors. Previous beliefs maintain that viral-induced tumors bear antigens common to all tumors induced by the same virus (47), while carcinogen-induced tumors have distinct antigens specific for each tumor (3). On the other hand, more recent evidence suggests that carcinogen-induced tumors may bear tumor-associated antigens which are common to other tumors induced independently by the same carcinogenic agent (48). Since preliminary evidence suggests some reactivity to allogeneic prostate TSA, perhaps additional refinements in the solubilization of tumor antigens could increase the sensitivity of skin responses to antigens common to allogeneic prostatic tumors, permitting diagnostic value in skin testing.

The specific response of a prostatic carcinoma patient to extracts of allogeneic BPH is difficult to explain. Although patients with other solid tumors (malignant melanoma [25], carcinoma of the breast [36], and carcinoma of the lung [38]) have shown reactivity to nonmalignant tissue from the organ originating the tumor, the patients responded to membrane extracts from the autologous neoplasm as well. Consequently, patients with other solid tumors may be immune to at least two antigens: a TSA and an organ-associated antigen, present in the benign organ of tumor origin. In the prostatic carcinoma patient reacting to allogeneic benign prostatic tissue, no cutaneous reactivity was observed in response to autologous or allogeneic solubilized components of prostatic carcinoma or to solubilized allogeneic blood cells from the patient donating the benign prostate. If this observation represents response to an organ-associated antigen, then two additional phenomena must exist to explain the patient's failure to respond to solubilized adenocarcinoma of the prostate. First, in the process of neoplastic transformation and differentiation the prostate cells must have undergone alteration or loss of their organ-associated antigens. Such a process must have taken place following sensitization of the host to these organ-associated antigens. Second, serum-blocking factors specific for the tumor-associated antigens must have abrogated the host's cutaneous response to the solubilized prostatic carcinoma. Equally possible is that reactivity was to HL-A antigens present in solubilized benign prostatic tissue, and that these HL-A antigens were less effectively solubilized by application of the extraction technique to whole blood cells of the same patient.

The absence of cutaneous reactivity to solubilized

components of autologous hyperplastic prostate brings to our attention the possibility that cells of abnormal tissue growth, though benign, could acquire neoantigens specific for that disease state. However, the possibility of neoantigens remains unresolved, as the benign prostate could constitute an immunologically privileged site (49), preventing sensitization of the patient to organ-specific antigens as well as to possible neoantigens of prostatic hyperplasia.

Several explanations can account for the skin response to autologous extract of hyperplastic prostate in a patient with stage B-1 adenocarcinoma. First, although the tissue examined was consistent with prostatic hyperplasia, the extracted tissue may have contained adenocarcinoma. Second, the neoplastic portion of the prostate may have disrupted the barriers, permitting the prostate to maintain immunologic privilege. The disruption of immunologic barriers would permit the host to be sensitized to TSA and/or organ-specific antigens. If a host could sensitize to organ-associated antigens, one might expect surgical manipulation to break down immunologic barriers and permit sensitization of the patients to their own benign glands. The observation that no patients with BPH reacted to extracts of autologous tissue makes this second possibility less likely. On the other hand, neoplastic tissue could act as an adjuvant, enhancing the immunizing capacities of antigens associated with BPH. Third, adenocarcinoma of the prostate could be multifocal in origin. Although the tissue obtained appeared histologically benign, malignant transformation may have been initiated at the subcellular level. Such early transformation could include the formation of neoantigens specific to prostatic adenocarcinoma. Solubilization of these neoantigens may have been responsible for the cutaneous response.

In conclusion, the observation that 40% of ten prostatic carcinoma patients responded to autologous tumor extract strongly suggests that adenocarcinoma of the prostate may bear TSA to which a host can respond. The lack of similar responsiveness in patients with BPH suggests that benign tissues do not bear distinct antigens or that the prostate is immunologically isolated from the host. Additional isolated observations accentuate the multiple components involved in host-tumor immunorelationships: common antigenicity between allogeneic tumors, multifocal subcellular neoplastic transformations, organ-specific antigens, serum-blocking factors, and sites of immunologic privilege. Although the in vivo assay of skin testing measures all three limbs of the cell-mediated immune system (afferent limb, central limb, and efferent limb) (46), variations in responsiveness further underscore the complexities of delayed hypersensitivity responses to TSA. Further resolution and refinements of the antigens extracted are required to develop skin testing to the level of clinical value.

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Studies on the Prostate and Testis as Immunologically Privileged Sites

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SUMMARY

The immunology of the prostate and testis was studied by observing allograft survival in rats and by measuring humoral antibody and delayed hypersensitivity responses to tissue antigens in the rabbit. The delayed hypersensitivity response to tissue antigen(s) was definitely diminished in those organs without demonstrable afferent lymphatics (ie, prostate, anterior eye chamber) while the humoral response was unaffected. Allograft survival in the rat showed prolonged survival in the testis and anterior eye chamber with intermediate survival in the prostate and muscle and abbreviated survival of orthotopic grafts. The extraordinary incidence of prostatic carcinoma with increasing age is discussed in light of these data. The apparent paradox of the testis as an immunologically privileged site remains unexplained.

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Our interest has centered on the immunology of the prostate and testis. Gittes and McCullough have hypothesized that the prostate is an immunologically privileged site because of its lymphatic anatomy, and that "latent" carcinoma of the prostate may be a consequence of this phenomenon (1). Other workers have suggested that the testis is also an immunologically privileged site (2). We have studied these organs and others in the rabbit and rat (1,3). We have confirmed for ourselves the excellent lymphatic drainage of the testis comparable to that found in skin or to a lesser degree in skeletal muscle, and the absence of conventional afferent lymphatics in the glandular prostate, similar to the absence in the anterior chamber of the eye.

We have attempted to relate these anatomic considerations to the immunology of these organs in two ways: (*a*) by using implanted skin allografts and recording survival in these five organs; and (*b*) by measuring the immune response using standard techniques to an isolated, particulate tissue antigen placed within these organs.

MATERIALS AND METHODS

Rats

Adult male (200 g) inbred rats of the Fischer 344 (Fi), Lewis (Le), and Buffalo (Bu) strains were obtained from Simonsen Laboratories, Gilroy, California. The strain combinations of Bu→Le and Le→Fi were chosen so that our data would represent an array of immunogenic barriers. The H-1 locus disparate Bu→Le combination represents a strong histocompatibility difference, and the Le→Fi combination represents an intermediate one (4).

Full-thickness skin grafts approximately 2 × 2 mm square were obtained from the ear of a Bu or Le strain donor. Grafts were transplanted either orthotopically or implanted into the anterior eye chamber, gluteal muscle, testis, or ventral prostate of each Le strain or Fi strain recipient. Control recipient strain autografts prepared by the same method were simultaneously implanted in the opposite paired organ. All techniques used have been described previously (5).

Orthotopic allografts were placed on the panniculus carnosus of the chest. Dressings were changed every 3 days until rejection was imminent and examined daily thereafter.

Based on information from preliminary experiments, Le→Fi allografts were removed with a portion of the surrounding tissue on the 26th and 38th days following implantation. Bu→Le allografts were removed on the 10th and 20th days. Specimens were fixed in alcohol and multiple sections of each specimen were stained with hematoxylin and eosin for histologic grading. Each specimen was assigned a grade of 1, 2, 3, or 4 as described previously (5), 1 representing histologically normal skin and 4 representing complete rejection.

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Rabbits

Adult male New Zealand white rabbits (2–3 kg) were used. Sheep red blood cell (SRBC) stromata were prepared by a standard technique (6). After blenderizing, SRBC stromata were suspended in 0.15 M NaCl with 1/10,000 merthiolate. Protein determination was done by the Folin technique. The immunizing solution was prepared by adding a 50-50 suspension of incomplete Freund's adjuvant in normal saline to the SRBC stromata to a protein concentration of 1.6 mg/ml. Each rabbit was then immunized by the appropriate route with 0.05 ml of this suspension (80 μg SRBC wall protein). Intradermal and intramuscular injections were given in a standard fashion. The prostate and testicle were exposed through a low midline incision under Innovar and ketamine anesthesia. Eye chamber injection was done under Innovar, ketamine, and topical anesthesia. A 25-gauge needle was inserted through the conjunctiva of the orbit into the anterior chamber. The anterior chamber fluid was then aspirated and the immunizing suspension injected. Control animals were injected intradermally with a 50-50 suspension of saline and incomplete Freund's adjuvant.

Animals were bled via ear vein on Days 8, 14, and 20 unless they were skin tested on Day 8 in which case they were bled only on that day. Skin tests were performed intradermally on the shaved flank with 0.3 mg SRBC wall protein suspended in 0.1 ml of normal saline.

Antibody titers were all done on the same day using the decomplemented test serum, a 1:20 dilution of fresh frozen guinea pig complement (previously absorbed against SRBC), and SRBC in a standard microtiter hemolysis technique. Titers were read as the highest well number showing complete hemolysis after 6 hours.

The delayed hypersensitivity skin response was maximal at 24 hours and documented by biopsy in several animals. The response was recorded as the mean diameter of induration of two measurements at right angles. Animals were skin tested on Days 8 and 21. No animal was bled for antibody determination after a skin test.

RESULTS

Rat Experiments

All grafts were noted to be grossly well vascularized at the time of biopsy. The control autografts showed uniformly excellent survival in all sites for the duration of the experiments. The orthotopic allografts in both the Bu→Le and Le→Fi strain combinations were completely rejected before there was a satisfactory histologic range of rejection seen in the implanted allografts. Orthotopic allografts exhibited abbreviated survival when compared to any of the implanted grafts ($P<0.001$).

The implanted allografts (ie, testis, muscle, prostate, anterior eye chamber) show a wide spectrum of histologic survival (figs 1–4). This is most evident in the less immunogenic strain combination (Le→Fi) (fig 1). Implanted allografts in all sites including the anterior eye chamber showed progressive rejection with time. This is impressively seen in the more immunogenic Bu→Le strain combination at 20 days (fig 4).

Allograft survival within alymphatic organs, namely the prostate and anterior chamber of the

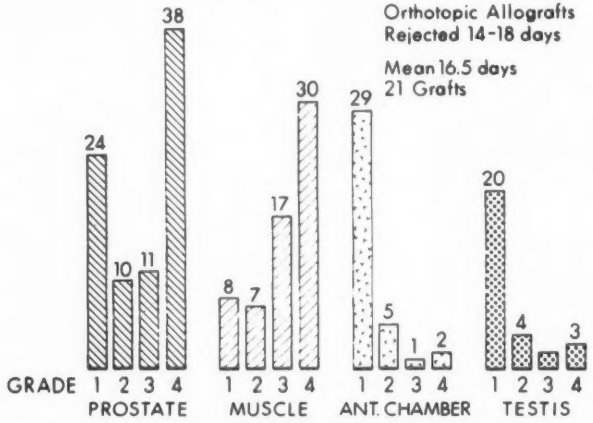

FIGURE 1.—Le→Fi skin allografts, 26 days.

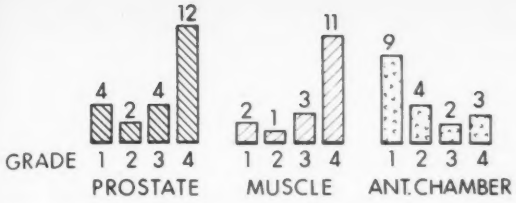

FIGURE 2.—Le→Fi skin allografts, 38 days.

eye, is not comparable. In contrast, allograft survival in the testis, well supplied with afferent lymphatics, is statistically as good as survival in the anterior chamber of the eye and much improved over survival in the prostate or skeletal muscle ($P<0.001$).

Rabbit Experiments

There was no correlation between route of immunization and hemolytic antibody levels. (In fig 5 each point represents the mean hemolytic antibody titer of six rabbits ± SEM.) Antibody was definitely present in animals immunized by all routes at 14 and 21 days, the titers increasing progressively over this interval. Apparent differences are not statistically significant.

Figure 6 shows the delayed hypersensitivity response to SRBC walls 24 hours following intradermal skin test. (Each point on the graph represents the mean diameter of induration recorded from six rabbits ± SEM.) At 8 days apparent differences were not statistically significant. At 21 days, however, the delayed hypersensitivity response of the animals immunized with SRBC walls injected into the prostate or the anterior chamber of the eye was definitely diminished when compared to the response of those immunized through organs with established afferent lymphatics (ie, testis, muscle, dermis) ($P<0.01$).

DISCUSSION

Afferent lymphatics, providing for the distribution of foreign antigen to local lymph nodes, are thought to play a key role in the usual set of events leading to cellular antibody formation (8,9). Cellular antibody, measured clinically as delayed cutaneous

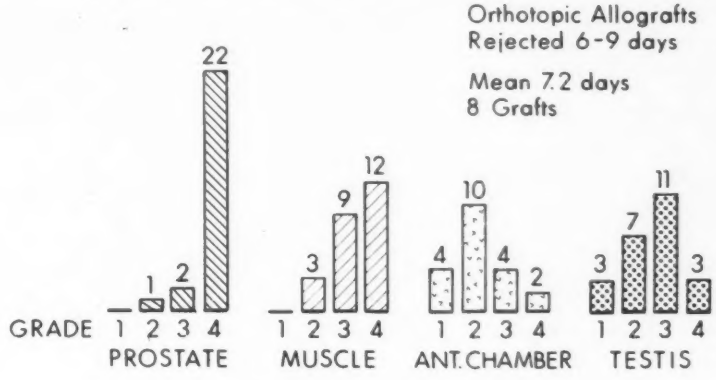

FIGURE 3.—Bu→Le skin allografts, 10 days.

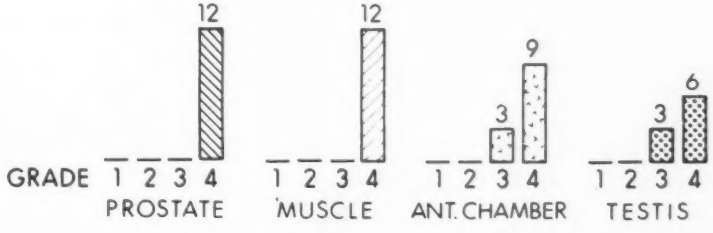

FIGURE 4.—Bu→Le skin allografts, 20 days.

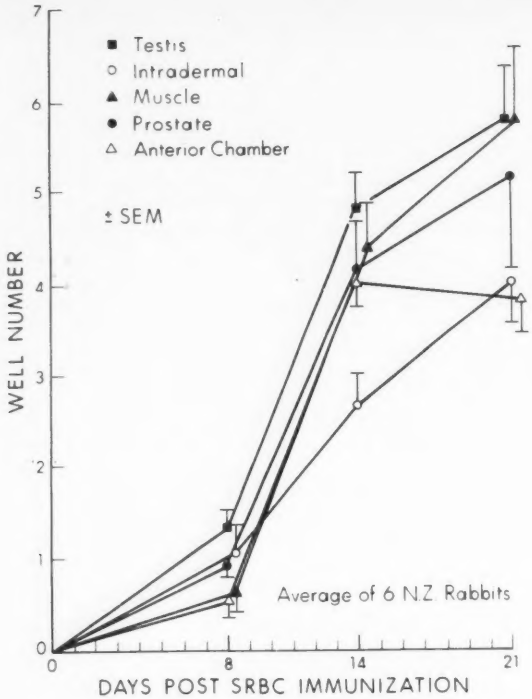

FIGURE 5.—Hemolytic antibody titer (recorded as highest well No. showing complete hemolysis).

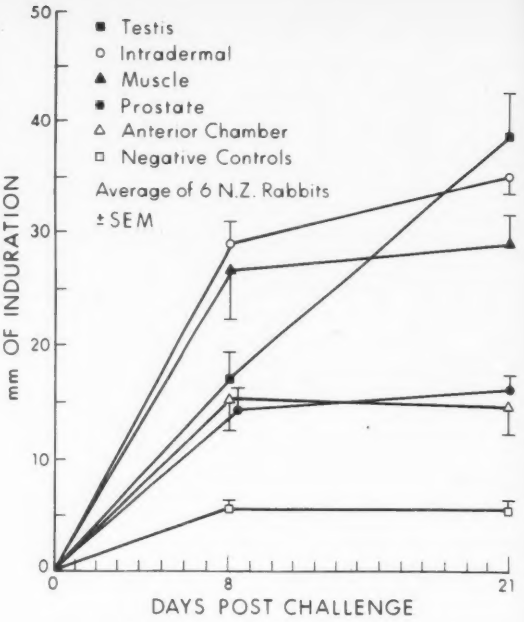

FIGURE 6.—24-hr response to intradermal injection of SRBC walls.

hypersensitivity, is an important mechanism in the rejection of foreign tissue (10).

In naturally occurring immunologically privileged sites, such as the brain and the anterior chamber of the eye, the absence of demonstrable afferent lymphatics has been thought to explain the prolonged first set survival of allografted tissue (11). In artificial immunologically privileged sites, such as the alymphatic skin island, a direct cause and effect relationship between the presence of intact afferent lymphatics and the primary immune rejection of allografted tissue has been demonstrated (8,12,13). However, in any of these examples subsequent challenge with a second allograft results in rapid rejection even when placed in a privileged site. Many authors have explained these findings by considering the absence of afferent lymphatics supplying local lymph nodes as a defect in the sensitizing limb of the immune response (1,8,11). This, they claim, may result in a primarily humoral antibody response, which could permit an established graft to survive while precluding the initial vascularization and hence the survival of a secondary allograft (1,8). Our experiment shows that the humoral response elicited from within the rabbit prostate was robust, but the cellular response was indeed decreased. Thus, the hypothesis that latent carcinoma may arise because of an oversight of immune surveillance and may be kept "latent" by a humoral response (1), remains viable after these experiments.

Of the several surprising results from our rat experiments, the most intriguing was the prolonged survival of allografts implanted within the testis. It was suspected that the excellent lymphatic drainage of the testis might be bypassing the lymph nodes and emptying directly into the venous system. Theoretically this might result in the same form of afferent immune blockade as seen in the alymphatic skin island. But our own study of the lymphatic drainage of the testes confirmed the work of others, showing excellent uptake by the paraaortic nodes (14).

This finding was inconsistent with earlier hypotheses and prompted us to look for some facts regarding the antibody response to tissue antigen(s) placed in the organs concerned. We measured the antibody reaction to an isolated antigen (SRBC walls) placed in the same organs in an animal with known immunogenic capability (rabbit). We recognized the pitfalls of combining data from different species. However, the antigens used were both cell

based, particulate, and grossly stationary. In addition, experimentally, immunologic differences between rabbits and rats are thought to be quantitative rather than qualitative.

With SRBC walls in the rabbit we were able to show a direct relationship between the presence of demonstrable afferent lymphatics and the strength of the delayed hypersensitivity response. Given what is known about this response, cellular antibody, and tissue rejection, we expected to see this result reflected in our allograft survival data in rats. Indeed we do, with the important exceptions of the prostate, where the survival is unexpectedly short (*see* ref 5), and the testis, where the survival is unexpectedly long.

The prolonged survival of allografts within the testis comes in the face of a very strong delayed hypersensitivity response to isolated antigen(s) placed in the testis of the rabbit. Afferent immune blockade is not a tenable explanation in this setting. Indeed, based on our data, there is no reason to suspect any deficiency in the systemic immune response, and we are left searching for local blocking factors within the testis itself.

The paradox of the testicle as an immunologically privileged site has not been explored. Recently, Dig-Kuri et al have had success with parathyroid allografts within the testis of the rat (2). Greene has shown that tumor heterograft survival in the testis is not prolonged in presensitized animals (15). This is consistent, as previously noted, with what is known about other immunologically privileged sites.

Temperature effects or other complex local immune mechanics may explain these findings. More attractive, however, is the possible role of the uniquely high tissue levels of steroid hormone(s), most notably testosterone, in the testis. It has been shown that there is an approximately 20:1 testicular interstitial fluid to plasma ratio of testosterone in the rat (16).

The immunosuppressive effects of pharmacologic doses of the glucocorticoid steroid hormones are well known. Although their mechanism(s) of action in this regard is poorly understood (17), it is widely confirmed that they can greatly inhibit the homograft reaction (18,19). Some of the immunosuppressive activity of these hormones is clearly bound to their glucocorticoid (eg, anti-inflammatory) effects. Their effects on the homograft reaction may not be.

Although testosterone is not a glucocorticoid hormone, it shares some characteristics with glucocorticoids. Novelli et al have shown that in large doses testosterone has benefits in preventing shock in rats equivalent to large doses of hydrocortisone and 6-methylprednisone (20). Testosterone suppression of in vitro lymphocyte blastogenesis has also been demonstrated in man (21).

Early experiments with testosterone in mice and rabbits have shown no immunosuppressive activity of testosterone (18,19). However, in these experiments the doses used, though pharmacologic, were probably insufficient to raise the plasma testosterone to the level of normal testicular interstitial fluid.

Other individual steroids, such as progesterone, or steroid combinations may account for our findings regarding the testis. However, afferent immune blockade associated with the absence of demonstrable lymphatic drainage appears to play an important role in the phenomena associated with other proven immunologically privileged sites.

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Preliminary Studies of Histologic Prognosis in Cancer of the Prostate [1]

M. Harada, F. K. Mostofi,* D. K. Corle, D. P. Byar, and B. F. Trump [2]

In a previous communication (1) the problem of grading carcinoma of the prostate was discussed in detail. Briefly stated, several systems of grading have been proposed; although each worked well for the investigator who proposed it, none was shown to be reproducible so that many pathologists have refused to grade carcinoma of the prostate. More recently, two classifications have emerged which have attracted considerable attention since they were based on a study of a large number of cases: (*a*) Gleason (2,3), and (*b*) Mostofi (4).

Evaluation of these two systems was carried out as a part of an extensive study of histologic and cytologic variables in carcinoma of prostate undertaken to determine if any one or a combination of a number of histologic or cytologic variables was of prognostic significance. The results of that study will be published later. In this presentation it is our purpose to report our observations in two areas: (*a*) a comparison of our use of Gleason's system with his own classification on the same cases, and our reproducibility of Gleason's system by repeat classification of the same slides; and (*b*) an evaluation of Mostofi's grading system.

METHODS AND MATERIALS

The slides of 2000 cases of carcinoma of the prostate from the first Veterans' Administration Cooperative Urological Research Group on file at the Armed Forces Institute of Pathology were available for study. All slides had been stained with hematoxylin and eosin stain. Followup information was available on all cases at the National Cancer Institute. Each case was classified according to the Gleason and Mostofi systems. Although we experienced certain difficulties in reproducing Gleason's system, these were, in part, overcome by two conferences with Gleason prior to initiation of the study. In 584 of these, Gleason had personally classified the cases but our reading was done without reference to his results. Our results were then compared to his.

For the Mostofi classification we had a double check since one column was specifically devoted to anaplasia (slight, moderate, marked) and a second column to grading based on anaplasia (1, 2, and 3); these could be checked against each other.

After the initial study and for reasons unrelated to this report we repeated the study on 798 cases without reference to our previous categorization. This is referred to as the Second Study and it served to demonstrate the degree of reproducibility of the systems by the same individual.

For evaluating the prognostic significance of these systems the statistic is computed on the number of deaths divided by total patient months of followup, expressed in deaths per 1000 patient months.

RESULTS

Reproducibility of Gleason's System

Our application of Gleason's system compared to that of Gleason was carried out in 584 cases (table 1). For the primary pattern there was agreement between us and Gleason in 64% of cases and disagreement in 36%. For the secondary pattern, agreement was obtained in 44% and disagreement in 56%. When the sums of our primary and secondary patterns were compared to those of Gleason, agreement was recorded in only 38%; however, in only 18.5% were the differences > 1.

The reproducibility of Gleason's system by the same individual on two different occasions was studied in 772 cases (table 2). Comparison of our initial study with the second study of the same slides showed reasonable agreement. In 81% of the primary pattern and 73% of the second pattern we obtained the same values. Other details are given in tables 1 and 2. The findings indicate that individuals who have mastered the Gleason system can reproduce their own readings with reasonable accuracy.

It will be recalled that Gleason has reported that the combined values of his primary and secondary categories and the stage of the tumor provided more

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TABLE 1.—Comparison of our results (AFIP) using Gleason's system with results of Gleason

Pattern	Agreement	Disagreement		Disagreement by > 1
		AFIP > Gleason	AFIP < Gleason	
Primary Nos.	373	129	82	53
%	63.9	22.1	14.0	9.1
Secondary Nos.	259	220	105	100
%	44.3	37.7	18.0	17.1
Combined Nos.	222	234	128	108
%	38.0	40.1	21.9	18.5

TABLE 2.—Comparison of our initial application of Gleason's system with our 2nd reading of the same cases

Pattern	Same	Lower on 1st reading	Higher on 2nd reading	Disagreement by > 1
Primary Nos.	625	123	24	17
%	81.0	15.9	3.1	2.2
Secondary Nos.	569	88	115	47
%	73.7	11.4	14.9	6.1
Combined Nos.	548	138	86	66
%	71.0	17.9	11.1	8.5

TABLE 3.—Comparison of death rates for categories based on our use (AFIP) of Gleason's system and his own

Category	AFIP		Gleason	
	Nos.	Death rate*	Nos.	Death rate*
4–6	22	9.8	43	8.2
7	32	6.4	32	8.0
8	57	8.4	91	8.5
9	139	11.6	131	12.9
10	89	12.2	47	15.1
11–12	185	20.7	151	19.4
13–15	60	29.9	89	26.5
All categories	584	13.8	584	13.8

*Death rates are expressed as deaths (from all causes) per 1000 patient months.

prognostic information than histologic evaluation alone. When this was done with our data (table 3) it was found that the death rates as obtained by our figures were quite comparable to those of Gleason's on the same cases.

Results Using the Mostofi System of Grading

The parameters used have been adequately described and illustrated in previous reports (4).

Gland formation and type of glands.—Death rates according to percentage of various gland patterns are presented in table 4. When the tumor consisted primarily of simple large glands or small glands the prognosis was good. On the other hand, as the percentage of microglands, fused glands, glands-in-glands, or nonglandular elements increased the prognosis became progressively worse. As we might expect the highest death rates were observed in those patients in whom a large percentage of the tumor was undifferentiated.

Size of nucleus.—The relationship of the size of the nucleus to death rate is shown in table 5. For each, the percentage of tumor cells with small, intermediate, and large nuclei is estimated. The percentage of small nuclei did not appear to be related to death rates and therefore the data were not presented in table 5. For medium and large nuclei the data have been arranged in percentile ranges in order to clarify the effects on the death rates and eliminate groups with small numbers of cases. The data presented in this table suggest that large nuclei are associated with worse prognosis than medium-sized nuclei, since if the tumor shows a large percentage of large nuclei it has a higher death rate but if > 40% of cells have medium-sized nuclei the prognosis is good.

Nuclear anaplasia.—Each case was classified as showing slight, moderate, or marked nuclear ana-

TABLE 4.—Death rates by % of glandular pattern

	% of tumor*									
	0		1–10		11–30		31–60		61–100	
Glandular pattern	Nr	DR	Nr	DR	Nr	DR	Nr	DR	Nr	DR
Simple large glands	535	6.88	196	1.99	60	2.18	7	7.87		
Small glands	31	20.16	63	16.88	142	7.77	319	5.23	243	1.18
Fused glands	440	3.81	118	4.38	146	7.74	76	7.09	18	11.33
Cribriform	430	4.44	166	4.67	144	6.03	46	7.99	12	11.10
Medullary, column, and cords	412	2.78	197	5.77	140	9.03	36	12.20	13	85.27

*Nr = Nos. of patients; DR = death rate. Death rate expressed as deaths per 1000 patient months using cancer deaths only.

TABLE 5.—Size of nucleus related to death rates

Size of nucleus	Level (%)	No. of patients	No. dead	Death rate
Medium	0–40	88	37	10.12
	41–80	601	171	5.22
	81–100	109	8	1.14
Large	0%	175	30	2.91
	1–30%	466	126	4.93
	31–100%	157	60	7.98

TABLE 6.—Nuclear anaplasia of predominant cell related to death rates

Degree of anaplasia	No. of patients	No. dead	Death rate
Slight	107	9	1.24
Moderate	564	144	4.51
Marked	127	63	14.88

plasia and death rates were calculated for each of these categories (table 6). It is apparent from the table that nuclear anaplasia has a profound effect on prognosis. Those with marked anaplasia had a death rate 12 times as great as those with only slight anaplasia. Comparison of data relating to nuclear anaplasia from the first study with those of the second study in the two columns, as mentioned above, showed minor variations.

CONCLUSIONS

Our study of reproducibility of Gleason's system shows that although exact agreement in identifying primary and secondary patterns either between ourselves and Gleason or with repeated readings by ourselves is far from perfect, we can, nevertheless, form groups of patients with prognoses quite comparable to those of Gleason. As might be expected the agreement with ourselves on repeat reading was greater than agreement between ourselves and Gleason.

Application of the Mostofi system revealed that nuclear anaplasia and glandular differentiation offered a simple and reliable method of prognostication. Time did not permit us to correlate histologic differentiation (gland formation and type of gland) and anaplasia with death rates or a combination of these and the clinical stage. These are now being prepared.

Further studies are also in progress to identify other variables or combinations of variables which may further improve our prognostic capabilities in carcinoma of the prostate.

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Scanning Electron Microscopic Study of Prostatic Cancer[1]

John F. Gaeta,[2,*] Jacob E. Berger,[3] and Mary C. Gamarra[3,4]

SUMMARY

Scanning electron microscopy is applied to the study of a 3-dimensional surface and organization of a normal prostate gland and of samples of prostatic disease, including five cases of benign hyperplasia and 15 cases of prostatic adenocarcinoma of different degrees of differentiation. The study provided further evidence regarding the nature and origin of prostatic cancer at a cytologic level and indicated alternate pathways to the neoplastic spread within the prostate gland.

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Previous histologic studies of prostatic cancer have provided substantial information about its general morphologic characteristics, distribution, and spread, but the inherent pleomorphic character of the disease and the lack of uniform criteria for classification have hampered many attempts to correlate and compare the existing body of data regarding results of treatment and differences in therapeutic modalities.

Some investigators (1) have attempted to characterize the early appearances of the disease, but whereas in other glands (endometrium, breast, intestine, etc) the concept of carcinoma in situ has been well established, an objective definition of the corresponding neoplastic stage in the prostatic gland has not been universally accepted.

In order to obtain more information about the nature of prostatic cancer, new techniques have been applied as they became available. Transmission electron microscopy (TEM) provided valuable knowledge of the ultrastructure of prostatic cancer (2,3), demonstrating some structural differences between normal and neoplastic prostatic cells; these differences appeared to be slight or minimal when comparing benign or hyperplastic epithelial cells to those of well-differentiated prostatic cancer (4).

Since its introduction in 1965, scanning electron microscopy (SEM) has proven to be a valuable tool for the study of the ultrastructural aspects of biologic specimens and probably superior to TEM because of the additional advantage of providing a 3-dimensional image of critical importance for the definition of cell surfaces and cellular interrelations. The present study represents an attempt to gain further knowledge of prostatic cancer in its early and advanced stages by the application of SEM.

MATERIALS AND METHODS

Samples of prostate glands were obtained from patients who were autopsied following neoplastic and other diseases unrelated to the prostate gland or, in the case of neoplastic diseases, showed no gross or microscopic evidence of prostatic involvement. Four controls were utilized. Samples of five prostate glands showing gross evidence of nodular hyperplasia were obtained from patients who underwent suprapubic prostatectomy. A microscopic diagnosis of benign prostatic hyperplasia was confirmed by conventional light microscopy, including direct observation and study of the portion of prostate gland immediately adjacent to the sample selected for SEM. A similar selection and sampling were made for the SEM study of prostatic adenocarcinoma, with ten cases representing autopsy samples of patients who died with prostatic cancer and five other cases in which the material was selected from

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pieces of prostate gland obtained by transurethral resection of patients with prostatic cancer who had undergone this surgical procedure for relief of urinary obstruction symptoms.

Preparation of Specimens

Samples of prostatic tissue were cut into slices about 2-mm thick, diced into 5–10-mm cubes, and immersed in fixative. Glutaraldehyde (3% in 0.05 M cacodylate buffer, pH 7.2, with 6.8% sucrose at room temperature) was used for immersion and perfusion fixation. The tissues remained in this fixative overnight and were then washed in distilled water to prevent the appearance of granular deposits. Then they were placed in osmium tetroxide (1% in 0.05 M cacodylate buffer, pH 7.2, with 1.5% potassium ferricyanide) for 2 hours at room temperature following the technique of Weiss (5).

Other samples were fixed in 10% buffered formaldehyde, left overnight, and postfixed in osmium tetroxide as outlined above. This procedure rendered the same or better results than glutaraldehyde fixation, some of the specimens showing less shrinkage and better contrast.

After graded alcohol dehydration, tissues were brought through to graded Freons and then dried at the critical point of Freon 13 in a Bomar (Bomar Co, Seattle, Wash) critical point apparatus. The dried tissues were then mounted on a carbon disk and placed in a desiccator. Twenty-four hours later, the tissue-bearing disks were placed in a Vacuum Evaporator and with the tissue rotating and rocking, a thin layer of gold was placed over its surface.

During the initial part of the study, some specimens were freeze-dried by immersion in liquid nitrogen followed by vacuum drying and coating, but this technique was later abandoned in favor of the critical point method because of better preservation of tissue elements. The samples thus prepared were viewed with an ETEC model (B-1) SEM. The micrographs were recorded on Polaroid P/N 55 film.

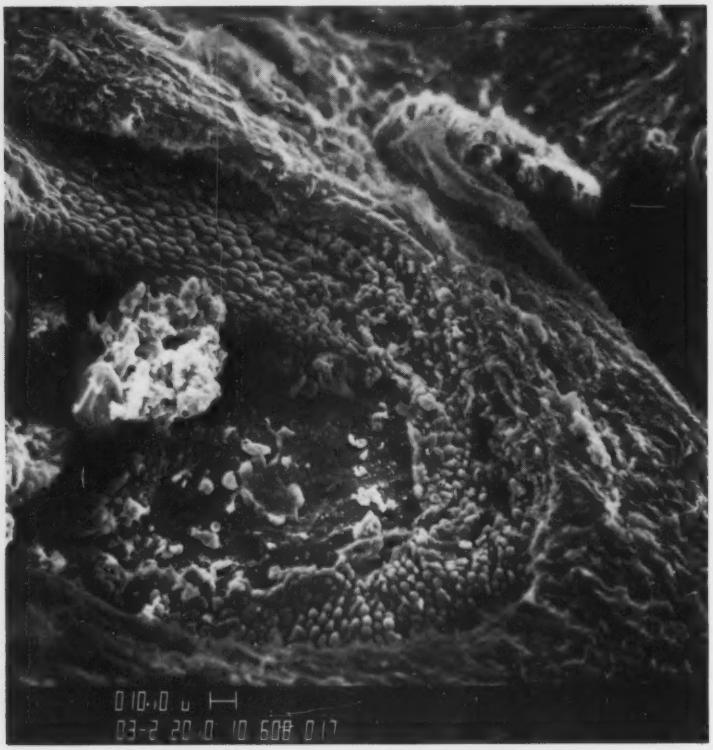

FIGURE 1.—View of prostatic duct surrounded by stroma. Note secretory material in lumen (× 300).

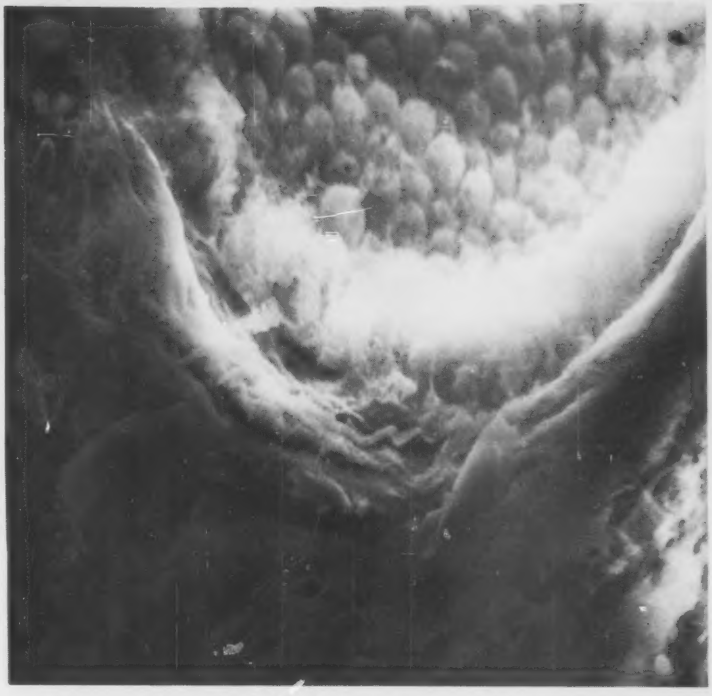

FIGURE 2.—Edge of prostatic duct. Note sharp boundary with stroma (× 400).

OBSERVATIONS

Direct observation of prostate gland controls correlated with the disciplined organization of the normal gland in which the presence of distinct tubular epithelial structures stands out clearly from the surrounding fibromuscular stroma (figs 1 and 2). A clear-cut distinction between acini and ducts was not possible on an organizational basis and differences between the two could only be made on the basis of size. The general arrangement was that of long tubules lined by a layer of round epithelial cells displaying marked uniformity. Except for occasional irregularities, most of them revealed the presence of short and delicate processes, sometimes ending abruptly and frequently connecting with similar processes in the adjacent cell (figs 3 and 4). A previous publication (6) also noted the presence of a small proportion of cells displaying an apical crater. Our controls confirmed the presence of cells with similar morphology (fig 5) although they did not seem to have any special geographic distribution and their relationship to the more prominent "microvillar" cells remains obscure. The prostatic stroma of the gland confirmed our concept of its fibromuscular nature, inasmuch as when viewed in magnifications > 1000 (fig 6) it showed the presence of two different types of fibers, some of them smooth, corresponding to smooth muscle, and others more characterized by a rough and irregular character.

Benign Prostatic Hyperplasia

As viewed by SEM, the image of prostatic hyperplasia disclosed a heterogenous group of changes, largely characterized by epithelial changes chiefly in the form of a significant increase in the size and exaggeration of the pattern of normal acinar and ductal structures. The general pattern and distribution of glandular structures in their relationship to the intervening stroma appeared generally preserved, but the intimate arrangement of the glands showed a significant departure from normal. At relatively low magnifications, many of the glandular units were irregular and their lumina were partially occluded by invaginations of smooth and blunt-

FIGURE 3.—Epithelial lining of prostatic acinus. Uniformity of cells all exhibiting microvilli (× 3500).

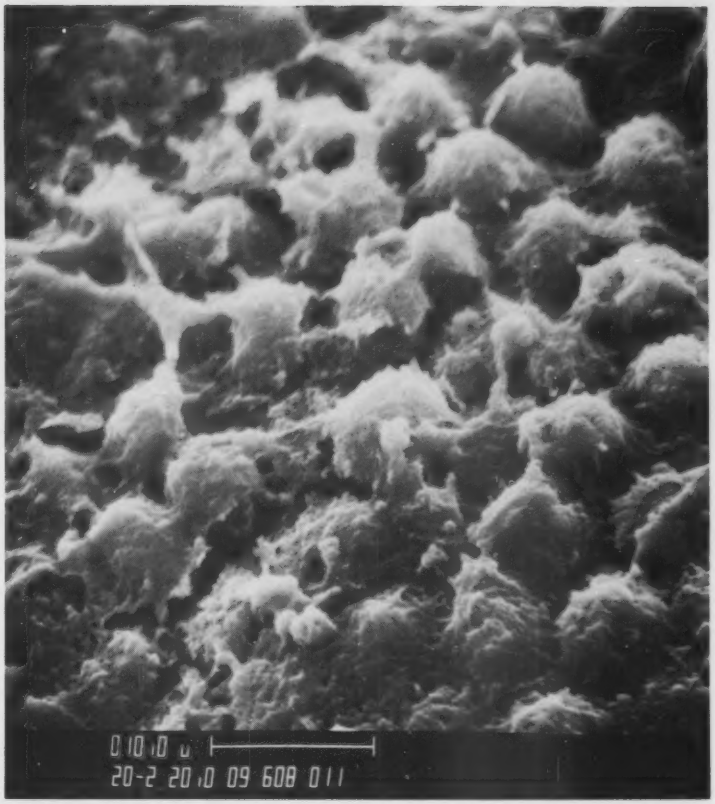

FIGURE 4.—Epithelial lining of prostatic acinus. Cells are uniform and show sharp villi connecting cell surfaces (× 2000).

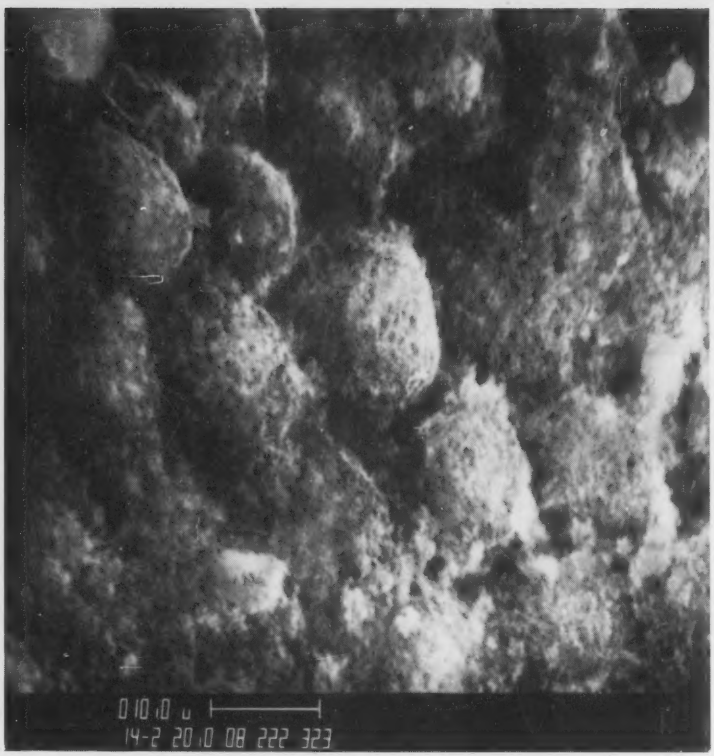

FIGURE 5.—Epithelial cells with short processes. Note crater cell in upper left corner (× 1400).

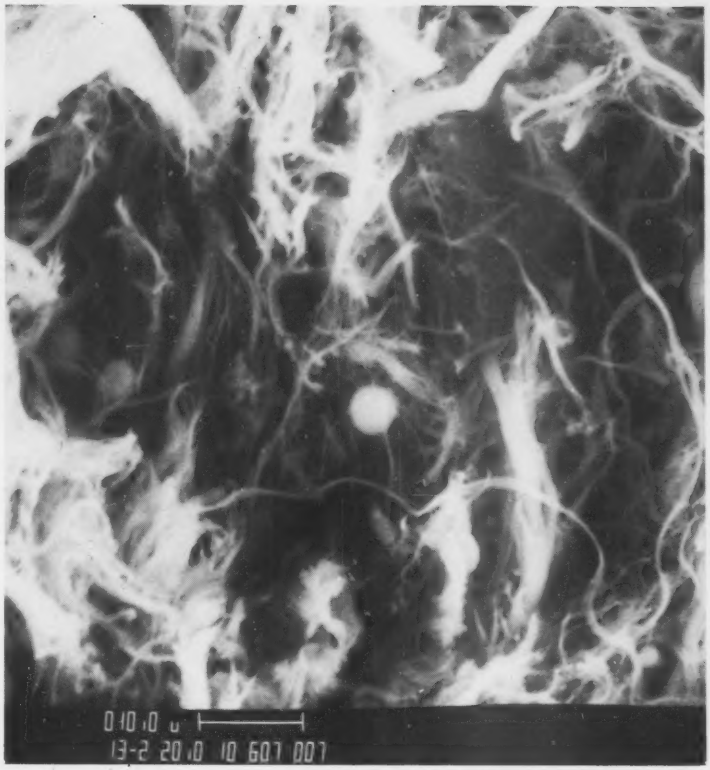

FIGURE 6.—Normal stroma of prostate. Note 2 types of fibers, collagen and smooth muscle (× 1300).

FIGURE 7.—Prostatic duct. Sample of benign hyperplasia. Uniform epithelial lining with abundant secretory material (× 500).

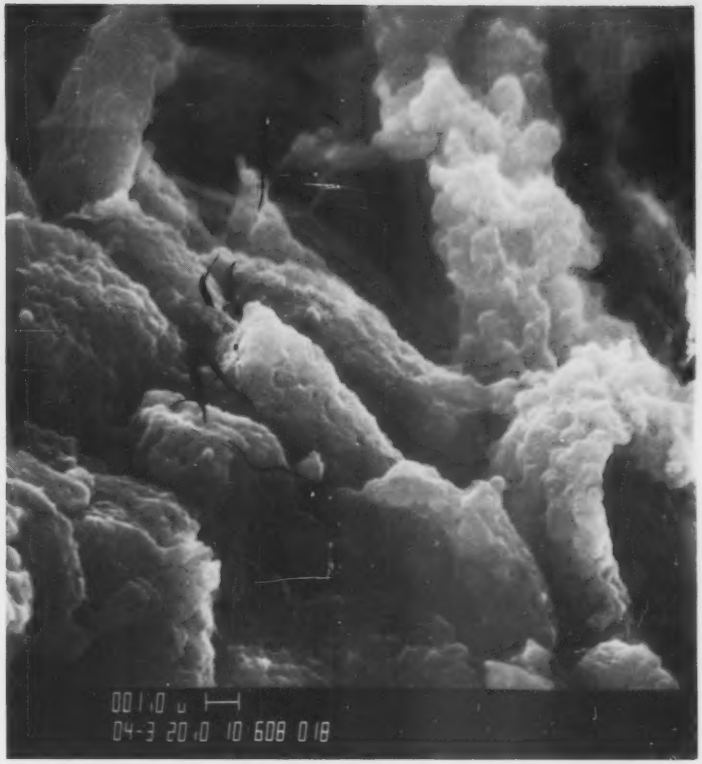

FIGURE 8.—Benign prostatic hyperplasia. Detail of ductal lining cells with granular surface and short villi (× 400).

ended villi. Others with similar organization (fig 7) also showed the presence of a significant secretory material, only occasionally seen in normal controls. Higher magnification of most specimens demonstrated an overall uniformity of the cellular population but showed a significant degree of variation among their intercellular relationships. This included a wide spectrum of morphologic changes ranging from ductal structures identical to those described in the normal controls to others largely characterized by a somewhat granular surface due to the presence of pseudovilli (fig 8), which were shorter than those described in normal cells.

Adenocarcinoma

Monitoring of light microscopy samples was carried out in order to include two groups of prostatic cancer samples for SEM study. The first group of samples included a pure population of malignant neoplastic cells invading stromal tissue and a second group of carcinoma samples contained a combination of neoplastic structures surrounding or invading hyperplastic or normal prostatic glandular tissue. In the former group, a special effort was also made to select an appropriate representation of carcinomas forming distinct glandular structures and others largely characterized by the presence of significant epithelial disorganization and anaplasia.

Examination of samples containing a single element of prostatic cancer disclosed the presence of a diffuse infiltration of the stroma by a population of cells largely characterized by lack of cohesiveness, and by capricious surfaces with uniform absence of either type of microvilli described in normal and hyperplastic cells (figs 9–11). Samples of prostatic carcinoma, regardless of their degree of differentiation, were frequently composed of an epithelial population of cells, displaying a smooth surface with sharp angles and hexagonal shapes (figs 12 and 13).

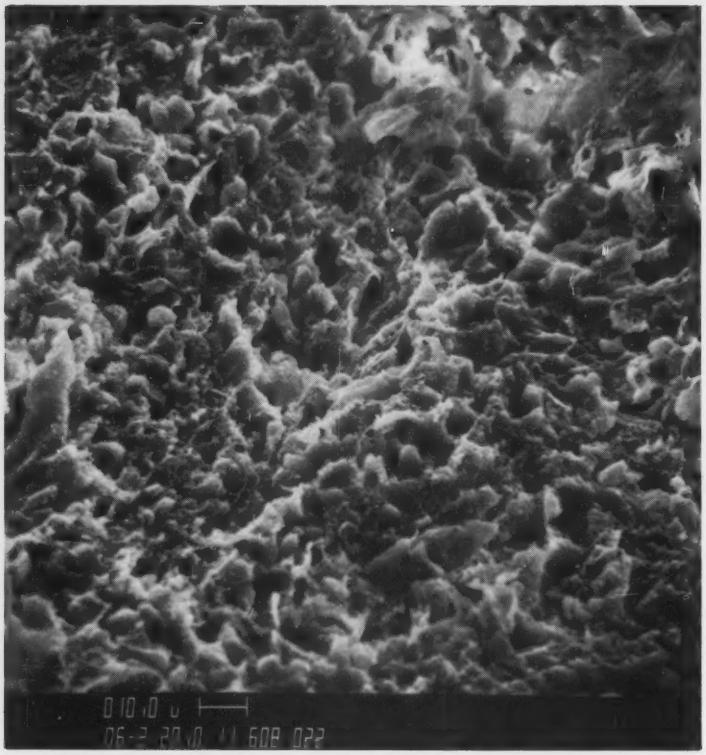

FIGURE 9.—Adenocarcinoma of prostate gland. Figure shows poorly differentiated type. Note diffuse growth of irregular cells and lack of boundaries with stroma (× 600).

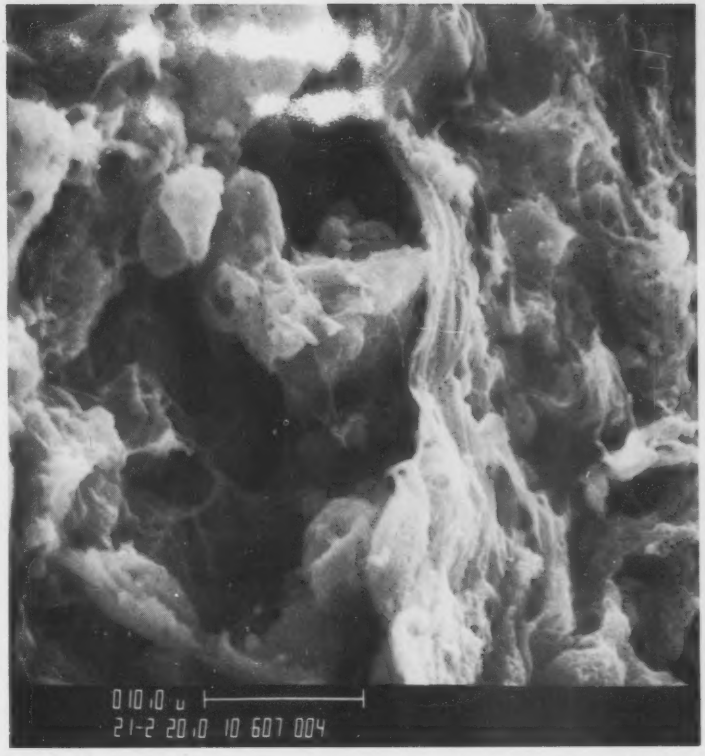

FIGURE 10.—Poorly differentiated adenocarcinoma. Cells exhibit irregular angles, lack of cohesion, and intimate contact with external fibers (× 2100).

FIGURE 11.—Poorly differentiated adenocarcinoma. Irregular clumps of tumor cells infiltrating frayed collagen fibers (× 1200).

FIGURE 12.—Moderately differentiated adenocarcinoma. View of neoplastic acinus. Note irregular and sharp surfaces of tumor cells and frequent lack of contact (× 1000).

FIGURE 13.—Moderately differentiated adenocarcinoma. Detail of single cell. Note sharp edges and surface pits (× 3500).

Those samples that included differentiating adenocarcinoma demonstrated characteristic crowding of glandular structures (fig 14) which, in closer detail, demonstrated circumscribed alveoli filled with irregular and angular epithelial cells, sometimes resting on a basement membrane, and frequently "floating" in neoplastic spaces with no visible attachments to surrounding elements (figs 15 and 16). The basement membrane of these neoplastic glands was frequently irregular and frayed (fig 17) and many of the tumor cells in direct contact with it showed further irregularities and distortion of their surfaces. Other neoplastic glandular structures showed more distorted shapes with lack of recognizable boundaries and tumor cells "spilling" over the stroma, indicating the early stages of diffuse infiltration (fig 18). Some of the samples also included examples of tumor growth in the form of cribriform structures which were clearly viewed by SEM as large and round nodules composed of a uniform cellular population with scattered "empty" holes, and surrounded by neoplastic cells (fig 19).

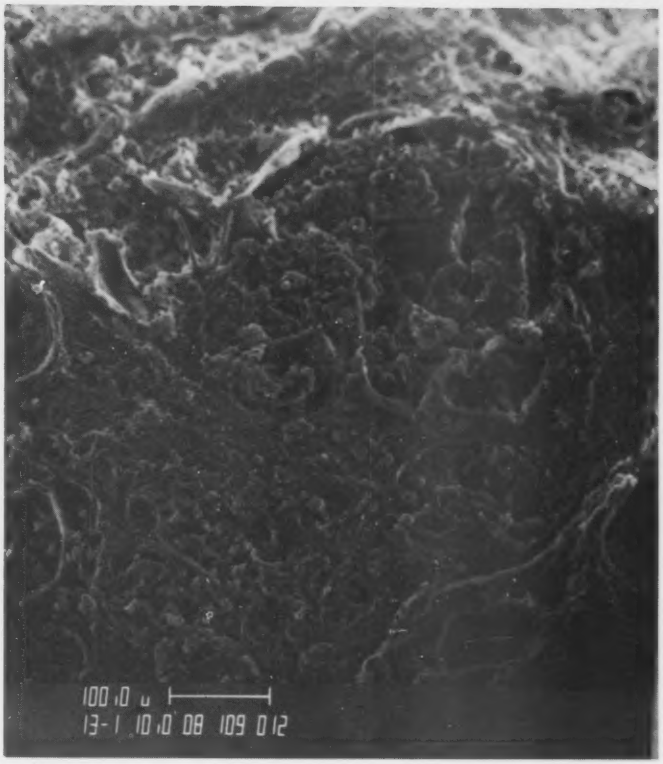

FIGURE 14.—Well-differentiated adenocarcinoma. General view of glandular pattern showing crowding of neoplastic glands (× 130).

FIGURE 15.—Well-differentiated adenocarcinoma. Neoplastic acinus. Note sharp demarcation with abnormal cells filling the lumen (× 500).

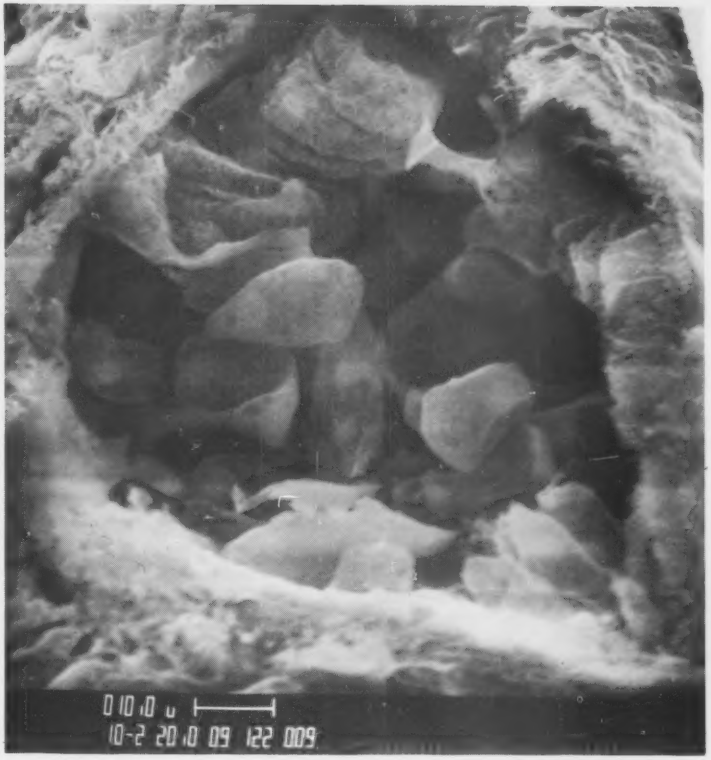

FIGURE 16.—Well-differentiated adenocarcinoma. Neoplastic acinus. Irregular cells with sharp angles and loose arrangement. Note irregular binding by slender processes (× 1000).

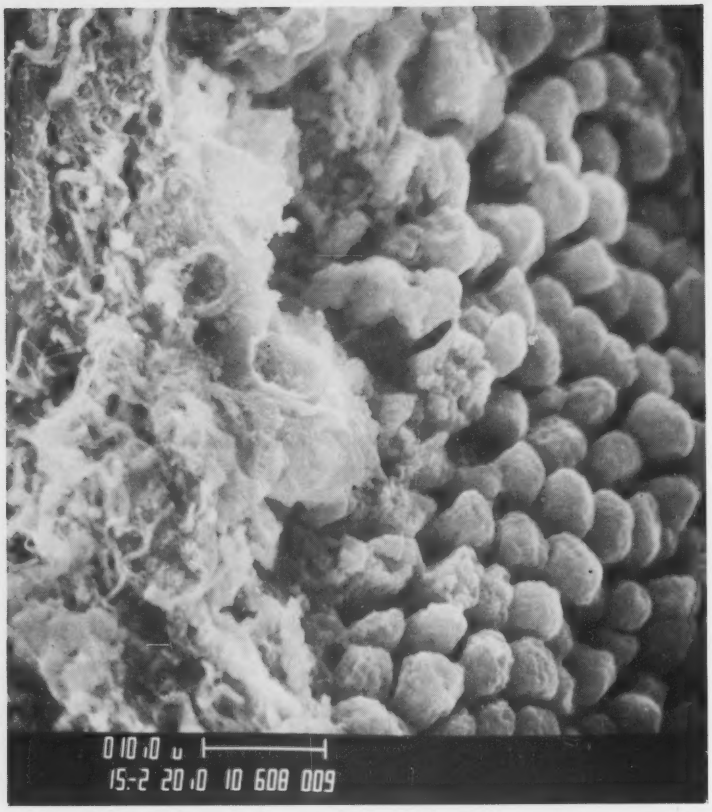

FIGURE 17.—Detail of neoplastic acinus. Note irregular characteristics of basement membrane (× 1500).

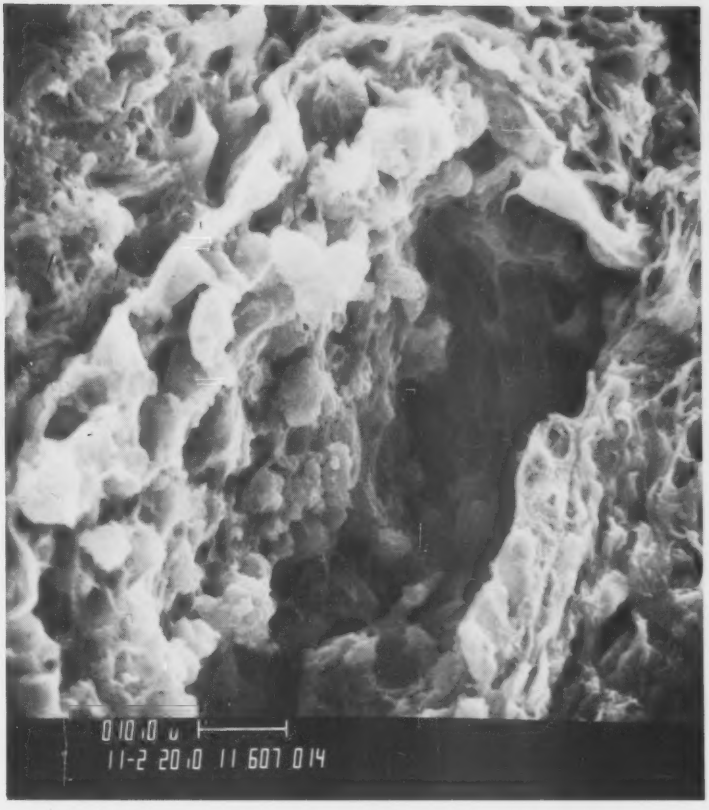

FIGURE 18.—Distorted neoplastic acinus showing lack of boundaries and "spilling" of tumor cells (× 1100).

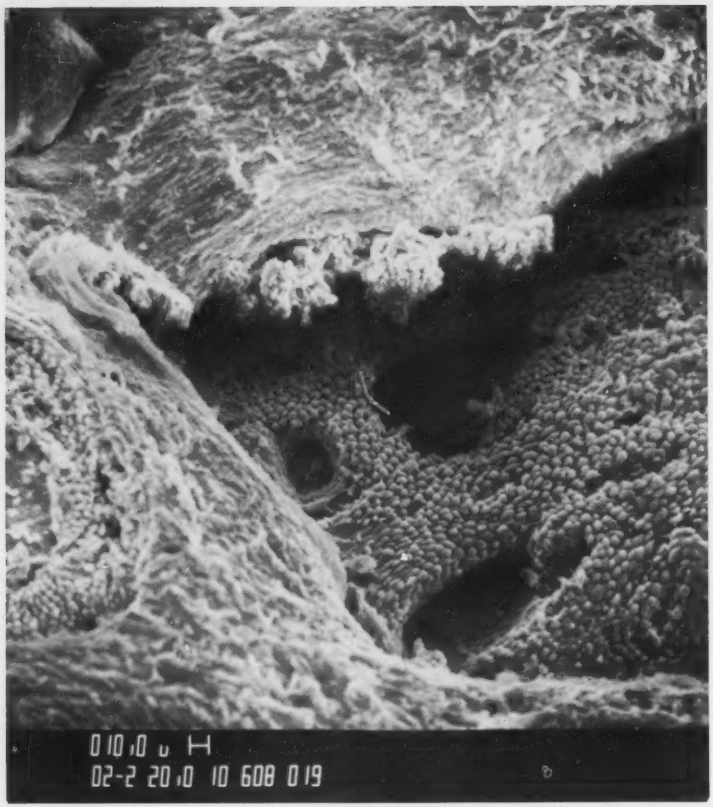

FIGURE 19.—Adenocarcinoma. Area of cribriform organization occupies right half of field (× 200).

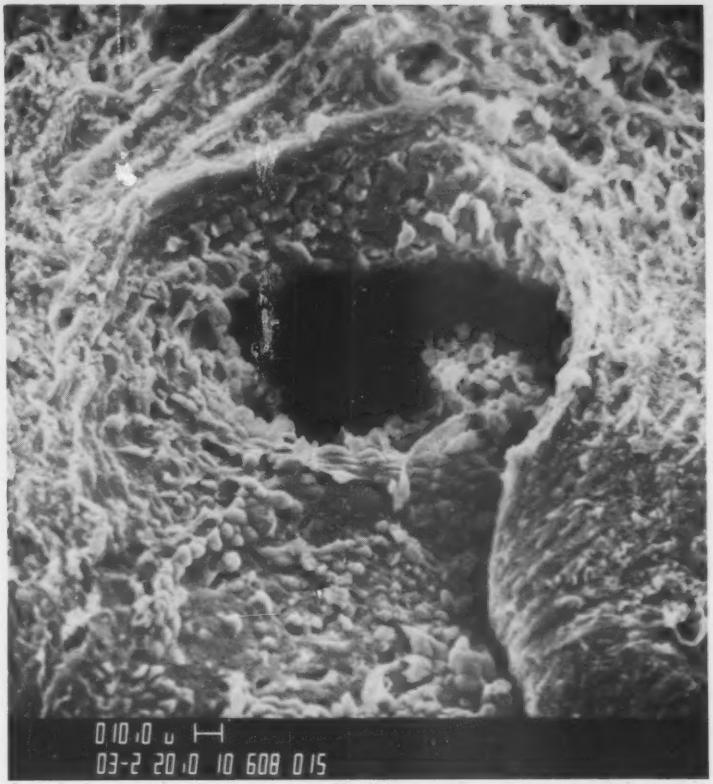

FIGURE 20.—Adenocarcinoma of prostate gland. Most of the duct is replaced by tumor cells; a small non-neoplastic projection of villous cells still remains at 6 o'clock (× 300).

SEM examination of prostatic samples, which under light microscopy included the interface between neoplastic and non-neoplastic prostatic structures, revealed areas of pleomorphic changes, sometimes difficult to interpret in regard to the exact boundaries between the two components. This was especially true when even low magnification demonstrated the presence of scattered glandular structures, partially populated by groups of epithelial cells, having surface characteristics similar in their villous arrangement to those described under benign prostatic hyperplasia (figs 20 and 21). The opposite side of the gland appeared more sparsely populated by a lining of irregular cells with no villous features and poor cohesive properties, frequently in continuity with similar irregular cells that appear to spread beyond the confines of the gland and into the adjacent stroma.

Other glands located within the interface of the two types of tissue as indicated by the corresponding image by light microscopy (fig 22) revealed similar features of two distinct cellular populations, the seemingly neoplastic population standing out and predominating against a background of epithelial cells (fig 23), disclosing a more cohesive arrangement and a more uniform character.

Finally, an occasional alveolar structure was formed (figs 24 and 25) displaying a uniform population of epithelial cells, well outlined by an intact basement membrane, and disclosing otherwise abrupt splitting of cytoplasmic membranes and sharp cytologic boundaries. Although initially interpreted as an artifact, their repeated presence in different specimens and their association with the more characteristic population of neoplastic and non-neoplastic structures within the same preparation suggested a different type of architectural organization.

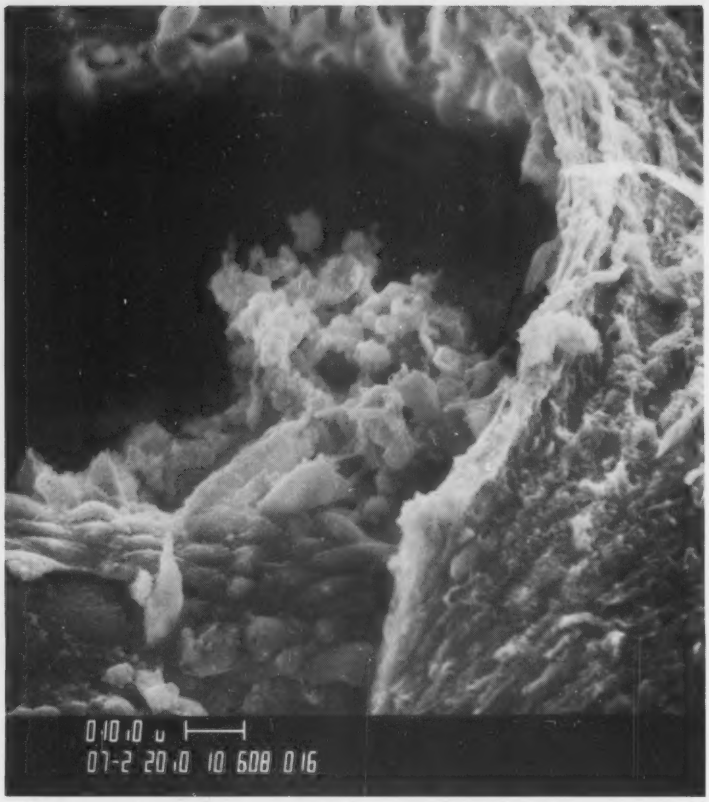

FIGURE 21.—Detail of fig 19. Note villous cells projecting into lumen (× 700).

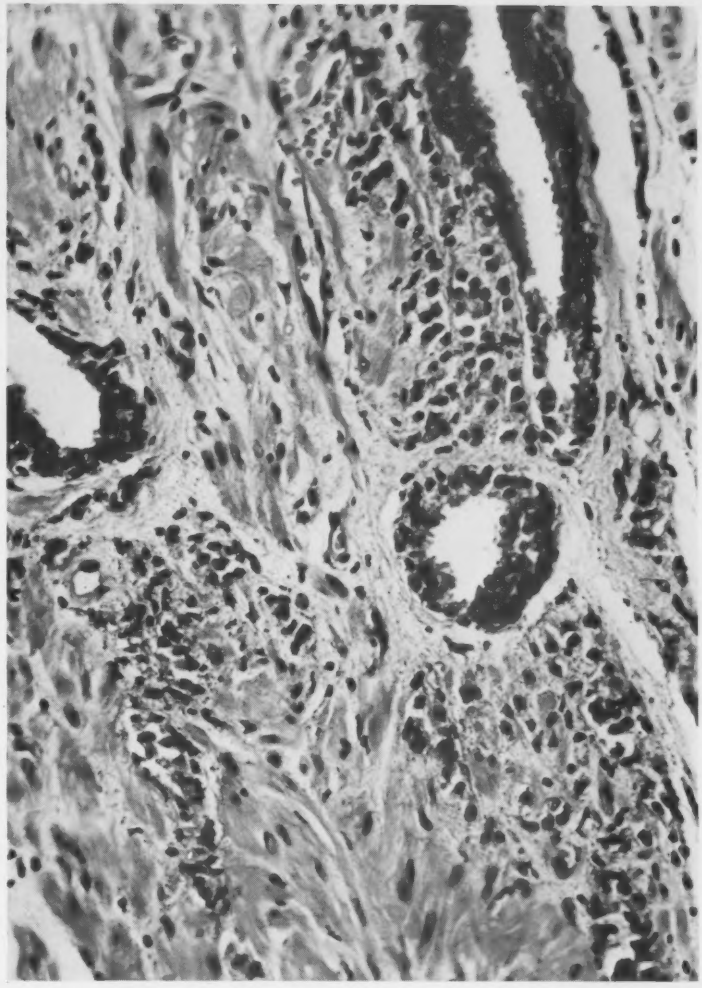

FIGURE 22.—Adenocarcinoma of prostate gland. Light microscopy field. Overview of interface of adenocarcinoma and non-neoplastic prostatic tissue (H & E stain, × 250).

FIGURE 23.—SEM view of sample in fig 22. Irregular glandular structure. Note 2 populations of cells within lumen (× 700).

FIGURE 24.—Glandular structure from advancing edge of differentiated carcinoma in fig 22. Uniform epithelial cells within boundaries of basement membrane. Note splitting of individual cells and early loss of attachments (× 900).

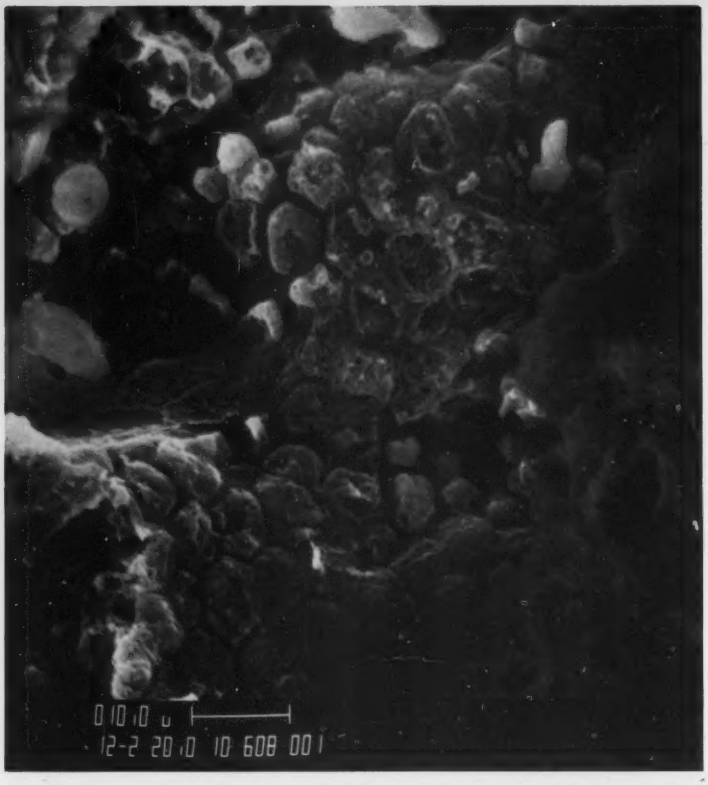

FIGURE 25.—Detail of glandular structure similar to fig 24. Note early pleomorphism and cellular detachment. Some of the epithelial cells show pits and crevices (× 1200).

DISCUSSION

In the present study, SEM was used to obtain information about the morphology of the prostate gland in benign and neoplastic disease. A 3-dimensional study of this gland provided valuable information regarding surface changes, largely because of the relative structural simplicity of this organ in which a reliable distinction of glandular and stromal elements can be made with ease. TEM studies in search of significant differences between benign and malignant prostatic disease proved unrewarding (2,4,7). Initial studies of prostatic changes by SEM have already given indications of more important changes at a structural and cytologic level (6,7). The same technique has also been applied to the study of neoplastic conditions of other organs with encouraging results (8,9).

The present findings corroborate the initial impression that the changes of prostatic hyperplasia are more of a quantitative nature, rather than representing a basic alteration of the glandular morphology. Clark et al (6) described the presence of occasional types of alveolar epithelial cells as part of the spectrum of hyperplastic changes, but we were unable to find any cytologic elements not already found in prostate controls.

Prostatic carcinoma, however, showed a much richer spectrum of surface changes which correlated well with the histologic changes found by light microscopy in each of the sections corresponding to the sample examined by SEM. Obviously, the general organization of the organ was markedly disturbed, and the relationship of gland-to-gland and of gland-to-stroma showed changes similar to those already seen and identified by light microscopy. More important was the recognition of different surface characteristics among the population of neoplastic epithelial cells, regardless of the degree of differentiation of the neoplastic field under study. The surface detail of anaplastic carcinomas (fig 9) lacked the definition observed in better differen-

tiated types, indicating a basic inability of anaplastic cells to grow along the tissue surface and their proclivity to become "buried" in stromal tissue. The most valuable single observation was certainly the recognition of surface changes at the level of neoplastic epithelial cells compared to non-neoplastic prostatic cells. Neoplastic cells consistently demonstrated complete lack of villous structures, irregular shapes, and surface pits and crevices (figs 10–13 and 16), even in samples of differentiated adenocarcinomas in which light microscopy showed little or no cytologic changes indicative of malignancy. Perhaps the earliest alteration appears to involve the cytoplasmic membrane leading to a disruption of the normal villous attachments, which normally maintain the cohesive organization of the normal ductal and acinar epithelia.

Some of the specimens studied included segments of prostatic tissue partially involved by carcinoma and they gave some insight into the 3-dimensional detail of the propagation of the neoplastic process within the gland. Some of the tubules (figs 20, 21, and 23) demonstrated partial invasion of the inner lining by a population of tumor cells in continuity with neoplastic cells outside the tubules. On the other hand, the presence of scattered acinar structures (fig 24), away from the areas of infiltrating carcinoma and still within the bounds of a well-defined basement membrane, lead to the impression that neoplastic extension along the epithelium of prostatic ducts occurs as a form of neoplastic propagation. This seems to indicate the possibility that prostatic cancer has two alternate pathways of extension within the gland, one in the form of massive infiltration with obliteration of glandular acini and ducts, and a second consisting of a gradual neoplastic transformation of prostatic ducts as they are approached by the advancing edge of the tumor. Further studies should explore the possibility that these two different types of spread of prostatic cancer may indicate differences in clinical behavior.

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Effects of Novel Acetylenic and Allenic Steroids on the Rat Prostate[1]

F. H. Batzold,* D. F. Covey, and C. H. Robinson[2,3]

SUMMARY

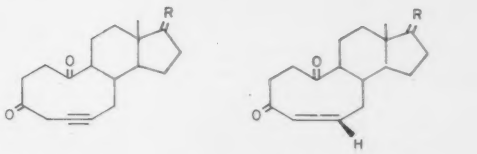
Novel acetylenic and allenic steroid analogs previously have been synthesized in this laboratory and were shown to be potent irreversible inhibitors of Δ^5-3-ketosteroid isomerase from *P. testosteroni*. This bacterial enzyme catalyzes the conversion of Δ^5-3-ketosteroids to the corresponding Δ^4-3-ketosteroids. These isomerizations are key steps in the mammalian biosynthesis of steroid hormones, and it was hoped that the acetylenic and allenic steroid analogs might inhibit androgen biosynthesis. We have now shown that these compounds cause a significant and dose-dependent decrease in the wet weight of both ventral and dorsal lateral prostate in the mature male rat.

[Cancer Treat Rep 61:255–257, 1977]

We have recently described (1–4) the synthesis of the novel acetylenic steroid analogs (I and II) and the derived allenic compounds (III and IV) (fig 1), and have shown them to be potent irreversible inhibitors of Δ^5-3-ketosteroid isomerase (EC.5.3.3.1) from *P. testosteroni* (5).

These compounds were designed as mechanism-based irreversible inhibitors of isomerase. Thus, the acetylenic ketones I and II are not themselves alkylating agents, but are substrates for the target enzyme, Δ^5-3-ketosteroid isomerase, which converts them to allenic ketones III and IV respectively. In contrast to the acetylenic steroids I and II, the allenic steroids III and IV are alkylating agents and are generated by the enzyme at the active site. Irreversible inhibition of the enzyme then occurs through covalent bond formation.

The bacterial Δ^5-3-ketosteroid isomerase catalyzes the conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids. Such isomerizations are key steps in the mammalian biosynthesis of steroid hormones, and it was hoped that the compounds I–IV might inhibit androgen biosynthesis. In this paper we describe the effects of these compounds on the wet weights of ventral and dorsal lateral prostate in intact mature male rats.



I, R=O

II, R=β-CH_3CO, α-H

III, R=O

IV, R=β-CH_3CO, α-H

FIGURE 1.—Structures of acetylenic steroid analogs (I and II) and the derived allenic compounds (III and IV).

Study in Intact Rats

Compounds I–IV were evaluated by the following protocol. Each pure compound was administered ip in propylene glycol (0.2 ml) daily for 7 days to intact mature Sprague-Dawley rats (250–300 g) at varying doses (1–20 mg/kg for compounds I and II and 0.8–8 mg/kg for compounds III and IV). On the eighth day the animals were killed and the ventral prostate, dorsal lateral prostate, seminal vesicles, testes, kidneys, liver, and adrenals were removed and weighed. Samples of each tissue were preserved for histologic examination.

[1]Supported in part by Public Health Service grant CA-16418 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[3]We thank Drs. D. S. Coffey and W. D. S. Heston and Miss S. Pivec for their advice and assistance.

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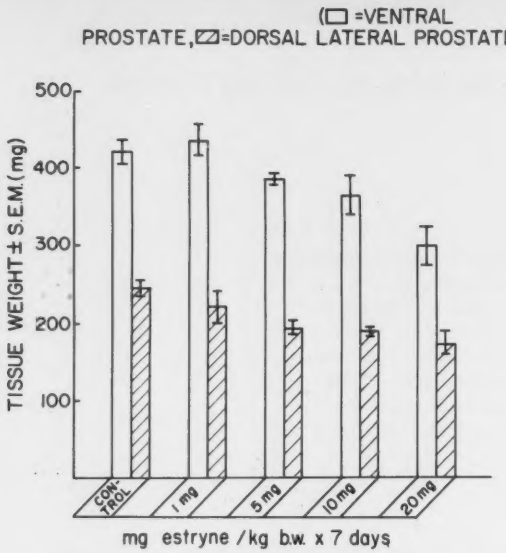

FIGURE 2.—Effect of 5,10-secoestr-5-yne-3,10,17-trione on the intact male rat.

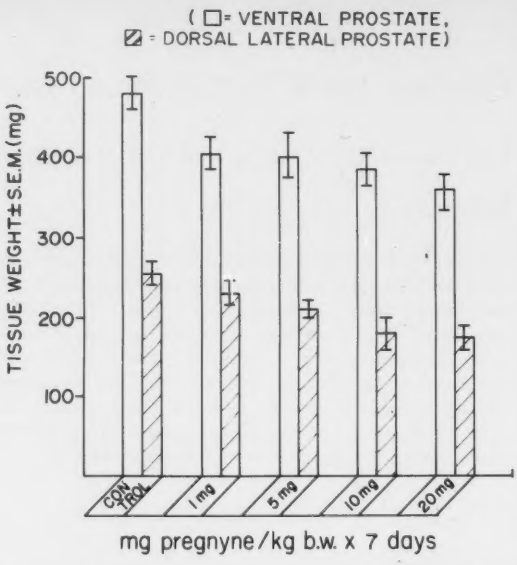

FIGURE 3.—Effect of 5,10-seco-19-norpregn-5-yne-3,10,20-trione on the intact male rat.

Results

The acetylenic steroid analog 5,10-secoestr-5-yne-3,10,17-trione (I) depressed the wet weight of both the ventral and dorsal lateral prostate in a dose-dependent manner when given at a daily ip dose between 1 and 20 mg/kg × 7 days (fig 2). Essentially no effect on the seminal vesicles, testes, kidney, or adrenal was observed up to the 10-mg dosage level. At the maximum dose of 20 mg/kg × 7 days the estryne caused a decrease of 25%–30% in the wet weight of the ventral and dorsal lateral prostate. This high dose also caused a decrease in the weight of the seminal vesicles and testes but not the adrenals.

The pregnyne (II) given at a dose of 20 mg/kg ip daily for 7 days also produced a 25%–30% decrease in the weight of both the ventral and dorsal lateral prostate. As was the case with the estryne (I), these effects were dose dependent (fig 3) with similar effects on the seminal vesicles and testes and with no effect on the adrenals at the highest dose. Both I and II, when given at a dose of 20 mg/kg × 7 days by ip administration, caused a 20% loss in body weight. This weight loss was not observed at lower doses. Non-drug-treated animals deprived of food until 20% of their body weight was lost, relative to controls, showed no significant change in prostatic weight.

The allenic ketone (IV) also caused a dose-dependent decrease in the wet weight of ventral and dorsal lateral prostate (fig 4). The highest dose given (8 mg/kg ip daily for 7 days) caused a reduction of 42% and 32% in the wet weight of the respective regions of the prostate. The wet weight of the seminal vesicles was similarly decreased. No weight losses were observed at any dose for the testes, kidneys, or adrenals. Loss of body weight and morbidity (25%) occurred at the highest dose.

On the other hand, the allenic ketone (III) caused only a 10%–15% decrease in prostatic weight even at a toxic dosage (8 mg/kg ip daily × 7 days).

Discussion

The synthetic steroids I, II, and IV each cause a dose-dependent decrease in the wet weight of both the ventral and dorsal lateral prostate of the intact mature male rat. Because the normal growth of the prostate is under endocrine control, it is susceptible to a variety of points of inhibition. The following sites of action were considered for the synthetic steroids under study:

(*a*) a direct estrogen effect, thus antagonizing androgen production;
(*b*) inhibition of luteinizing hormone production, thus removing the stimulus to the testes for androgen biosynthesis;
(*c*) direct competition with androgen at the target tissues; and

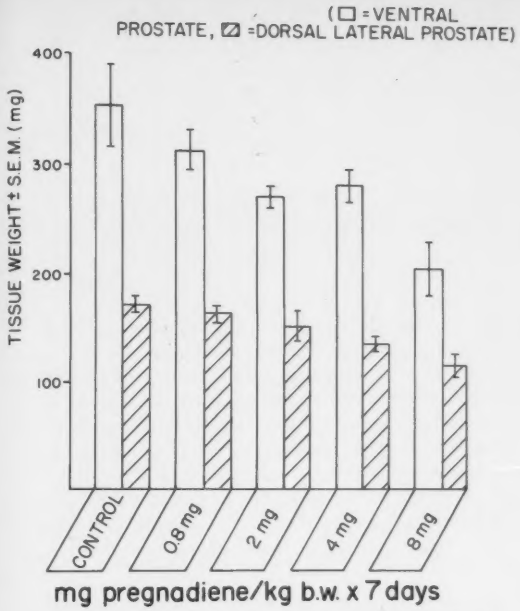

FIGURE 4.—Effect of (4*R*)-5,10-seco-19-norpregna-4,5-diene-3,10,20-trione on the intact male rat.

(*d*) inhibition of androgen biosynthesis, possibly by inhibition of steroid-transforming enzymes.

Direct estrogenic action by the estryne (I) seems unlikely. Immature female mice receiving I at a total dose of 100 μg showed no increase in uterine weight, whereas a maximum response to estradiol was seen at a total dose of 0.05 μg.

Preliminary studies in our laboratory using hypophysectomized rats suggest that significant inhibition of luteinizing hormone is not occurring.

Other studies in castrated rats using compound I suggest that, at least in this case, the action of exogenous testosterone propionate is not blocked. Direct competition with androgen at the target tissue in vivo thus seems unlikely in the case of acetylenic compound I.

The effects of the synthetic steroid analogs I, II, and IV could be explained by inhibition of androgen biosynthesis, perhaps through effects on Δ^5-3-ketosteroid isomerase. However, further work is in progress in our laboratory in order to further test this explanation.

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New Agents for Prostatic Cancer Activated Specifically by Prostatic Acid Phosphatase [1,2]

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SUMMARY

Potential cytotoxic compounds (spindle poisons) are being designed for the treatment of prostatic carcinoma, using the structural requirements of the substrates for prostatic acid phosphatase (PAP). Colchicine has been modified in ring C to give colchiceinamides of substituted ethanolamines and ethanolaminephosphates. Another new series of compounds modifying ring B of thiocolchicine have been prepared. Three *O*-phosphates of the thiocolchicine series have also been made. One has been examined for its specificity toward PAP. Some toxicity data of these compounds in mice have also been reported. Colchiceinamide-(L)-ephedrinephosphate has been examined in stumptail monkeys and some preliminary results are reported here.

[Cancer Treat Rep 61:259–263, 1977]

Since the prostates of man and the larger primates (table 1) have very high prostatic acid phosphatase (PAP) activity, and since a high percentage of prostatic cancer also has high PAP activity, we are attempting to design possible chemotherapeutic agents for prostatic cancer based upon enzyme activation specifically by PAP. This rationale has been reinforced by our earlier observation (1) that PAP has special structural requirements that enable us to design phosphate substrates that will be hydrolyzed specifically by PAP as compared to other acid and alkaline phosphatases in the body. Two structural features that give specificity to substrates for PAP are a certain degree of steric hindrance and the presence of a nitrogen, especially substituted nitrogen in the molecule. For this reason, phosphorylcholine and D-ephedrinephosphate turn out to be very specific for PAP (1,2). Although phosphorylcholine is hydrolyzed by alkaline phosphatase at alkaline pH, it is very poorly hydrolyzed by other acid phosphatases at acid pH as compared to PAP. The rate of hydrolysis by the human prostate is 1000 times faster than the human kidney cortex. Lundquist (3) indicated that phosphorylcholine is pro-

TABLE 1.—Estimated ratio of PAP activity per milligram of prostate as compared to that of rat after 3 hrs of incubation with phosphorylcholine at pH 4.8

Species	Ratio
Hamster	0.02
Mouse	0.20
Rabbit	0.25
Rat	1.0
Cat	1.5
Squirrel monkey	1.5
Guinea pig	2.1
Dog	60
Rhesus monkey	130
Stumptail macaque monkey	680
Baboon	1100
Man	1200

[1]Supported by Public Health Service grants CA-16077 and CA-02478 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Dedicated to the late Arnold M. Seligman, MD, American Cancer Society Professor of Research Oncology and Cell Biology, who pioneered this rationale of prostatic cancer chemotherapy through enzyme activation and under whose direction this work was done.

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TABLE 2.—Ratio of hydrolysis rates of human prostate and kidney cortex (P/K) with some selected phosphate substrates*

Compound No.	Substrate	P/K ratio	P/K_{Alk} ratio
1	$(CH_3)_3N^+CH_2CH_2OPO_3Ca\ Cl^-$ (phosphorylcholine)	1000	200
2	$OHCH_2CH(OPO_3H_2)CH_2OH$ (β-glycerophosphate)	250	820
3	$NH_2CH_2CH_2OPO_3H_2$	255	75
4	$NH_2C(CH_3)_2CH_2OPO_3H_2$	420	70
5	$NH_2CH_2CH(CH_3)OPO_3H_2$	460	75
6	$NH_2CH(CH_3)CH(CH_3)OPO_3H_2$	630	120
7	$NH_2CH(CH_3)CH(Ph)OPO_3H_2$	31	170
8	L-$CH_3NHCH(CH_3)CH(Ph)OPO_3H_2$ (L-ephedrinephosphate)	865†	220†
9	D-$CH_3NHCH(CH_3)CH(Ph)OPO_3H_2$ (D-ephedrinephosphate)	1050	920
10	Colchiceine-$NHCH_2CH_2OPO_3Na_2$	500	170
11	Colchiceine-$NHCH(CH_3)CH(CH_3)OPO_3Na_2$	780	1010
12	Colchiceine-$N(CH_3)CH(CH_3)CH(Ph)OPO_3Na_2$	980†	2400†

*Rates of hydrolysis on a per milligram wet weight basis were obtained at pH 4.8 for acid phosphatase and at pH 9.0 for alkaline phosphatase of kidney. The results are given as the ratio of prostate to kidney (P/K). The normal incubation time is between 1.5 and 3 hrs unless otherwise stated.
†Since the rate of hydrolysis is slow, longer incubation time was necessary (24 hrs).

duced in the seminal vesicles, transported into the semen where it is found in large amounts, and then, after ejaculation, is hydrolyzed by PAP. He was not aware of its high order of specificity for PAP (mainly present in the secretory vacuoles [granules] of prostatic epithelial cells) (4–6) as compared to lysosomal acid phosphatases of other tissues. The role of nitrogen and steric hindrance in giving high P/K ratios (rate of hydrolysis by human prostate compared to the rate by human kidney) is illustrated with a few selected compounds in table 2. Phosphate compounds were made using the spindle poison colchicine and the P/K ratios have been determined. Another parameter we consider important in designing and selecting possible useful agents is the maximum tolerated dose (MTD) ratio. This is the ratio of the MTD in male mice of the phosphorylated derivative to the MTD of the unphosphorylated compound (MTD-PO_4/MTD-OH). It was expected that the larger the MTD ratio the better the therapeutic index for prostatic cancer. Based on these two parameters (P/K and MTD ratios) it should be possible to develop compounds which could be used for treatment of prostatic carcinoma.

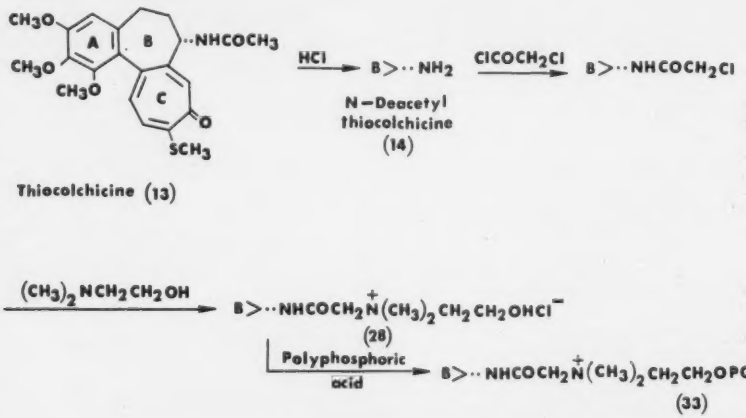

FIGURE 1.—Synthesis of thiocolchicine-ethanolaminephosphate.

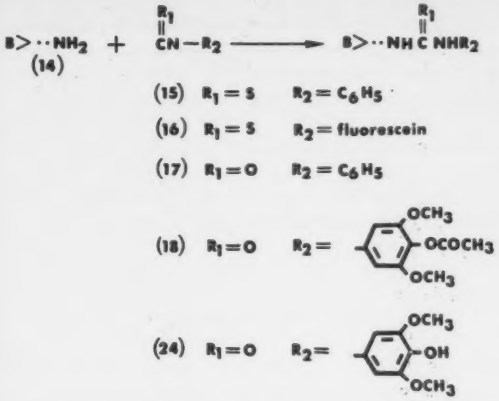

FIGURE 2.—Synthesis of thiocolchicine-ureas and -thioureas.

MATERIALS AND METHODS

The substituted ethanolaminephosphates (compounds 4–9, table 2) were prepared according to the procedure described by Cherbuliez and Rabinowitz (7) and Cherbuliez et al (8). Phosphorylcholine, β-glycerophosphate, and ethanolaminephosphate were purchased from the Sigma Chemical Co (St. Louis, Mo). Compounds 10–12 (table 2) were synthesized according to a previous report (1). Thiocolchicine was deacetylated with hydrochloric acid to give *N*-deacetylthiocolchicine (compound 14, table 3) (9). All thiocolchicine compounds having ring B modifications were made from compound 14, as described in figures 1–4. A detailed procedure for the synthesis of all the compounds along with their physical properties will be published. The P/K ratios (tables 2 and 4) of the substrates were done according to the procedure previously described (1). After the enzyme hydrolysis of the substrates, the free phosphoric acid was quantitatively determined using Fiske-Subbarow reagents (10). A Klett colorimeter was used.

RESULTS AND DISCUSSION

The P/K ratios of different substrates are reported in table 2. The importance of substitution(s) on nitrogen in giving substrates specificity for PAP is demonstrated by comparing the results of phosphorylcholine (compound 1) with that of ethanolaminephosphate (compound 3), and again by comparing two ephedrinephosphates (L and D, compounds 8 and 9) with *N*-desmethylephedrinephosphate (compound 7). Although substitution(s) on nitrogen did improve the specificity of the substrates for PAP compared with other acid phosphatases in the kidney, it did not behave the same way when PAP activity was compared with the activity of alkaline phosphatase of the kidney. This deficiency could be overcome when the model compounds 3–8 were reacted with colchicine at ring C to give colchiceinamide-substituted ethanolaminephosphates, eg, compounds 10–12, which, having a bulky group on the

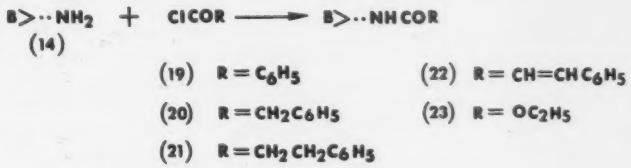

FIGURE 3.—Synthesis of thiocolchicine derivatives.

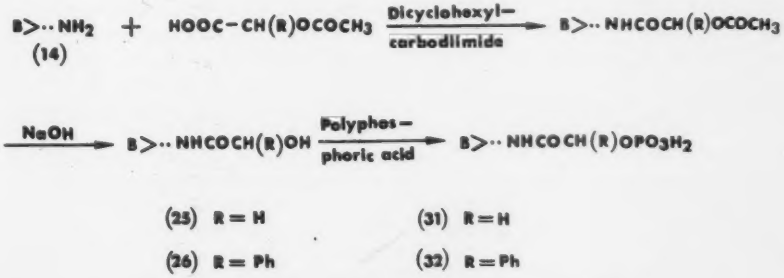

FIGURE 4.—Synthesis of thiocolchicine-glycolamidephosphates.

TABLE 3.—MTD in male mice of analogs of deacetylthiocolchicine (TC)*

Compound No.	Structure	MTD (mg/kg)
13	Thiocolchicine	3
14	Deacetylthiocolchicine	150
15	TC-NHCSNHC$_6$H$_5$	12
16	TC-NHCSNH-fluorescein	18
17	TC-NHCONHC$_6$H$_5$	10
18	TC-NHCONH-C$_6$H$_2$(3,5-OCH$_3$,4-OAc)	80+
19	TC-NHCOC$_6$H$_5$	25
20	TC-NHCOCH$_2$C$_6$H$_5$	9
21	TC-NHCOCH$_2$CH$_2$C$_6$H$_5$	30
22	TC-NHCOCH=CHC$_6$H$_5$	30
23	TC-NHCOOC$_2$H$_5$	30+
24	TC-NHCONH-C$_6$H$_2$(3,5-OCH$_3$,4-OH)	75+
25	TC-NHCOCH$_2$OH	4
26	TC-NHCOCH(Ph)OH	9
27	TC-NHCOCH$_2$N(CH$_3$)CH$_2$CH$_2$OH	23
28	TC-NHCOCH$_2$N$^+$(CH$_3$)$_2$CH$_2$CH$_2$OH Cl$^-$	75+
29	TC-NHCOCH$_2$N(CH$_3$)CH(CH$_3$)CH(Ph)OH (from L-ephedrine)	15
30	TC-NHCOCH$_2$N(CH$_3$)CH(CH$_3$)CH(Ph)OH (from D-ephedrine)	60+

*Compounds were dissolved in water with a minimum amount of alcohol and were administered by ip injection. Young male white mice, weighing about 35 g, were used for MTD determinations.

TABLE 4.—Thiocolchicine (TC) derivatives containing phosphoryl group

Compound No.	TC phosphates	P/K ratio*	P/K$_{Alk}$ ratio*	MTD† (mg/kg)	MTD-PO$_4$‡ / MTD-OH
31	TC-NHCOCH$_2$OPO$_3$Na$_2$	178	210	12	3
32	TC-NHCOCH(Ph)OPO$_3$Na$_2$	—	—	65	7
33	TC-NHCOCH$_2$N$^+$(CH$_3$)$_2$CH$_2$CH$_2$OPO$_3^-$Na	—	—	100	1

*Rates of hydrolysis by human prostate as compared to that of acid and alkaline phosphatase of human kidney cortex (P/K).

†In male mice.

‡Ratio of MTD of phosphorylated derivative to MTD of unphosphorylated derivative.

nitrogen, gave a high P/K$_{Alk}$ ratio. Proper steric hindrance also gave the substrates a good degree of specificity for PAP. This could be observed from the P/K ratio results of compounds 3–6. Simple methyl substitution(s) at α and/or β position(s) of ethanolaminephosphate (compound 3) to give compounds like 4, 5, or 6, gave higher values of P/K ratios. Using substitution(s) on nitrogen and the proper steric hindrance in the substrate, a good number of compounds with high P/K ratios were made. These compounds were then reacted with thiocolchicine at ring B. One compound, 33, of this series was made (table 4 and fig 1).

As mentioned earlier, the second parameter of these compounds for clinical usefulness is the MTD ratio (MTD-PO$_4$/MTD-OH), with a higher ratio indicating a better therapeutic index for treatment of prostatic cancer. In a previous paper (1) we reported colchicine compounds with ring C modifications, where a high P/K ratio was obtained but the highest MTD ratio was found to be 4, with colchiceinamide-(L)-ephedrinephosphate (compound 12). Although we suspected that the ratio should be nearer to 10, we gave compound 12 in an increased dose iv and later ip to a stumptail macaque monkey. The total dose to this monkey was about 2 g with the first dose being 50 mg iv three times a week and the last dose being 300 mg ip. The period of treatment was about 1 month. Total weight loss was about 13 lb from 25 lb starting weight. No significant histologic changes

were seen in the prostate which weighed about 1.9 g when the monkey was killed. A severe peritonitis was produced in this monkey by the irritating phosphate derivative (colchiceinamide-[L]-ephedrinephosphate). A second stumptail macaque monkey received 5 g of this compound given iv in 300-mg and, finally, 400-mg doses three times a week. No more than 2 lb in weight was lost during a period of 8 weeks. The monkey was killed, and the prostate gland weighed 3.0 g. Although the liver, kidney, lung, and spleen looked normal histologically, there was some evidence of damage to the epithelial cells of the prostate gland as compared to that of a normal monkey. Two more monkeys received iv doses of 100 and 200 mg respectively, three times a week for 8 weeks. When the monkey was killed, no appreciable change was noticed in the prostate. Further investigation of this work is in progress. Colchiceinamide-(D)-ephedrine was made and its MTD was found to be 25, making it equally toxic to the L-isomer, with an MTD of 25. Since compound 9 had higher P/K ratios than the L-isomer (compound 8), and since colchiceinamide-(D)-ephedrine and colchiceinamide-(L)-ephedrine were equally toxic, colchiceinamide-(D)-ephedrinephosphate is expected to be a better compound than colchiceinamide-(L)-ephedrinephosphate for clinical trial in monkeys. An attempt to synthesize colchiceinamide-(D)-ephedrinephosphate is in progress. Also, a ring B modification of thiocolchicine was done and compounds were made (figs 2–4); their MTDs are reported in table 3. Initially, compounds 13–23 were prepared without the free hydroxy group to be tested for MTD. Hydroxylated compounds 25 and 26 were made corresponding to the compounds having the lowest MTD (or highest toxicity). It has been noted that normally there is not much difference in MTD between the compounds with or without hydroxy (eg, compounds 13 and 25; 20 and 26). This way a better MTD ratio could be expected. Seven hydroxylated compounds (24–30) of thiocolchicine using ring B were made, and their MTDs are reported in table 3. Three of the compounds (25, 26, and 28) were phosphorylated with polyphosphoric acid to give compounds 31, 32, and 33, respectively. Their MTD ratios are listed in table 4 along with the P/K ratios of *N*-deacetylthiocolchicine-glycolamidephosphate (compound 31). The P/K ratios of compounds 32 and 33 are in progress. So far, of all the compounds made, the highest MTD ratio obtained was 7, with compound 32. If the P/K ratios of this compound are > 500, its clinical usefulness can be evaluated in monkeys.

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Androgen Receptors in Human Prostatic Tissue: A Review[1]

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SUMMARY

Methods for the measurement of androgen receptors in the human prostate have been reviewed. The differentiation of binding to receptor from binding to a contaminating serum protein, testosterone-estradiol binding globulin (TeBG), has been the major problem in the establishment of a reliable assay in man. Charcoal adsorption and Sephadex gel filtration (G-25) have been the simplest methods utilized, but unfortunately they do not eliminate binding to TeBG. Although other methods such as sucrose density gradient centrifugation, ion-exchange chromatography, and protamine precipitation are more specific for the measurement of the androgen receptor, they have not been uniformly reproducible and are too elaborate for easy clinical applicability. For clinical purposes, assays using potent synthetic androgens that do not bind to TeBG or anti-steroid antibodies may prove to be the methods utilized in the future to measure the androgen receptor content of human prostatic tissue.

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The concept that steroidal hormones mediate their effect through binding to intracellular proteins (receptors) has gained wide acceptance. Based on this principle, the endocrine sensitivity of both experimental tumors (1) and human breast cancer (2) has been related to the presence of cytoplasmic receptor proteins. Indeed, measurement of estrogen and progesterone receptors has been shown to be of value in assigning therapy in metastatic carcinoma of the breast (3). Similarly, the hormonal responsiveness of prostatic cancer may be related to the presence of cytoplasmic receptor proteins that bind androgen.

In 1968 Bruchovsky and Wilson (4) and Anderson and Liao (5) showed that following the administration of ^{3}H-testosterone to rats, the principle metabolite isolated from prostatic nuclei was ^{3}H-dihydrotestosterone (DHT). A two-step mechanism for the nuclear uptake of DHT has been proposed. Initially, testosterone is converted to DHT by the membrane-bound enzyme, NADPH-dependent 5α-reductase. DHT is then bound to a specific intracellular cytoplasmic macromolecular protein, the receptor. The steroid-receptor complex next undergoes a temperature-dependent physical change enabling the complex to be transported to the nucleus. There the complex is bound to acceptor substances on chromatin thereby activating transcription and resulting in the formation of messenger RNA and increased protein synthesis (6).

The androgen receptor has been studied most extensively in the rat ventral prostate. On sucrose density gradient centrifugation, the cytosol receptor sediments as an 8S. It is completely destroyed by heating to 50°C for 1 hour and is converted to a 3S–4S form by the addition of 0.4 M KCl. The receptor is completely precipitated by 30% ammonium sulfate or by the addition of polycations like protamine sulfate. Flat molecules with double bonds that extend in conjugation from ring A to B to C (eg, 17α-methyl-17β-hydroxy-estra-4,9,11-triene-3-one) bind with high affinity (Kd = 0.6×10^{-9} M) to the receptor. The receptor has an acidic isoelectric point (pH = 5.8), is eluted from DEAE cellulose with buffers of high ionic strength, and is unstable in the absence of DHT (6).

In man, the characterization of the androgen receptor has been complicated by the presence of a contaminating serum protein, testosterone-estradiol binding globulin (TeBG), that is not present in the

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TABLE 1.—Methods using gel filtration or charcoal separation

Author	Ref	Method	n	Kd	Remarks
Mobbs	8	Charcoal assay	2300 fm/g tissue	4×10^{-10} M	Steroid specificities more suggestive of TeBG. Cyproterone acetate did not inhibit binding but estradiol did. Estrogen treatment caused an increase in binding. Muscle had 1500 fm/g by same technique.
Walsh	9	Micro-Sephadex columns	3000 fm/g tissue	2×10^{-9} M	High-affinity binding seen in human prostate but similar binding seen in non-target tissue.
Geller	10	Incubation of tissue slices with ^{3}H-testosterone at 25°C; ammonium sulfate fractionation followed by Sephadex G-50 filtration of 0–33% fraction	—	—	Receptor peak seen in void volume. However, this was inhibited > 65% with 17β-estradiol. Peak not seen with skin and muscle. Thin-layer chromatography of peak showed 80%–90% as DHT.
Fang	11	Ammonium sulfate fractionation followed by Sephadex G-100, G-150, G-200 filtration	—	—	9.5S peak seen in 30%–50% fraction. No peak seen in 0–33% fraction. Steroid specificity shown. Was not inhibited by antiplasma antibodies. Excluded from G-150; retained by G-200 (vs refs 13,14).
Hansson	12	Incubation of benign prostatic hypertrophy slices at 3°C for 24 hrs followed by Sephadex G-100 filtration	—	—	Receptor peak seen in void volume: TeBG eluted 1 tube later. No tissue specificities or steroid specificities reported.
Mainwaring	13	Sephadex G-200 chromatography	—	—	Receptor peak seen in void volume (vs ref 11).
Hansson	14	Tissue slices incubated with ^{3}H-DHT or ^{3}H-testosterone at 37°C for 60 mins followed by Sephadex G-200 filtration	600 fm/mg protein	7×10^{-10} M	Peak indistinguishable from TeBG on ^{3}H-DHT incubations. On incubation with ^{3}H-testosterone, peak seen in void volume (vs ref 11), which was heat labile. Thin-layer chromatography showed most of radioactivity due to DHT. No steroid or tissue specificities shown.
Cowan	15	Sephadex G-25 and LH-20 filtration	—	—	In all samples of benign prostatic hypertrophy analyzed, binding to receptor indistinguishable from binding to TeBG. Steroid specificities similar to TeBG.
Rosen	16	Sephadex G-150 filtration	—	—	Receptor excluded from gel (*see* tables 2 and 4 also).
Geller	17	Whole tissue incubation followed by duo-gel filtration	—	—	Molecular weight of receptor 150,000 (vs refs 11,12,13). TeBG had same elution pattern as receptor. Differentiation between receptor and TeBG based on fact that receptor inhibited by cyproterone acetate and TeBG (plasma) not affected. Binding was significantly inhibited by 3α-androstanediol, estradiol, and 5β-DHT. Only 60% of the radioactivity in the peak was DHT.

rat. This protein has a sedimentation coefficient of 4.5S and binds with high affinity (Kd = 0.7–0.9 × 10^{-9} M) to DHT. If tissue is contaminated with as little as 4% serum, TeBG contributes approximately 2000–3000 femtomoles (fm) of high-affinity binding. TeBG is precipitated by 50% ammonium sulfate, is eluted from DEAE cellulose at pH 7.4 with buffers containing low salt concentrations, and is not precipitated by polycations such as protamine sulfate. Compared to the receptor, TeBG is heat stable.

The quantitation of receptor proteins is based on their ability to bind to radioactive ligands of high specific activity. In order to characterize this binding as specific for a cytoplasmic receptor, four criteria should be satisfied: (*a*) hormonal specificity, (*b*) tissue specificity, (*c*) high affinity, and (*d*) saturability (7). In this manuscript we have summarized the various methods that have been utilized in evaluating androgen binding in the human prostate, and have applied the above criteria in an attempt to determine whether this binding has been specific for the measurement of androgen receptors.

RESULTS AND DISCUSSION

A variety of methods have been utilized for the evaluation of androgen binding in human prostatic tissue. In some instances, these techniques have involved rather nonspecific methods for the identification of "receptor binding," eg, Sephadex G-25 or charcoal separation. In other instances, more specific techniques have been utilized, eg, Sephadex G-200 filtration, sucrose density gradient centrifugation, or ion-exchange chromatography.

Methods Utilizing Gel Filtration or Charcoal Separation (Table 1)

Following the incubation of DHT with the cytosol of human prostatic tissue, charcoal adsorption or micro-Sephadex columns (G-25) provides the most convenient and simple method for the identification of high-affinity binding. Utilizing a charcoal assay, Mobbs et al (8) have identified high-affinity binding (Kd = 4 × 10^{-10} M) in large quantities (2300 fm/g of tissue) in human prostatic tissue. However, because this binding was not inhibited by cyproterone acetate and because similar amounts (1500 fm/g) of high-affinity binding were present in muscle, these data suggested that the charcoal assay was not sufficiently specific to distinguish binding to TeBG from binding to an androgen receptor. We have reported similar results using G-25 micro-Sephadex columns (9).

In an attempt to develop more specific techniques for the measurement of androgen receptors, a variety of methods have been used to purify the receptor: (*a*) ammonium sulfate precipitation (10,11), (*b*) fractionation with Sephadex G-100-G-200 (11–16), and (*c*) suppression of DHT binding with cyproterone acetate (17). When ammonium sulfate precipitation was used, Geller and Worthman recovered "receptor" from the 0–33% fraction (10), whereas Fang and Hsu (11) demonstrated it in the 30%–50% fraction. Using Sephadex G-100 filtration, Hansson et al demonstrated high-affinity binding in the void volume of prostatic cytosol (12). However, as TeBG eluted one tube later, the distinction between receptor and TeBG was not clear. Geller et al attempted to improve separation by a duo-gel filtration technique (17). The elution patterns for receptor and TeBG were similar but the receptor peak was depressed by the addition of cyproterone acetate whereas the TeBG peak was not.

These techniques have provided some evidence for a specific receptor in human prostatic tissue. However, because TeBG is present in concentrations approximately 100-fold greater than measurable androgen receptor and because these methods fail to quantitatively eliminate binding to TeBG, they can not be adequately interpreted.

Methods Utilizing Density Gradient Centrifugation (Table 2)

Of all the techniques that have been utilized for the identification of androgen receptors, sucrose density gradient centrifugation appears to be the most specific. When prostatic cytosol is incubated with DHT and then analyzed by sucrose density gradient centrifugation, two distinct peaks of radioactivity are demonstrated: an 8S–9S peak and a 4.6S peak (18). On incubating the 0.4 M KCl extract of crude prostatic nuclei, a solitary 3S peak is seen. Because the 9S and the 3S peaks can be displaced by the simultaneous addition of 100-fold excess nonradioactive DHT or cyproterone acetate, it is assumed that these peaks represent high-affinity binding. In addition, incubation with radioactive testosterone, 3α-androstanediol, or 17β-estradiol does not produce a 9S or a 3S peak of radioactivity. However, these steroids do produce a 4.6S peak of radioactivity which represents binding to TeBG. After heating to 45°C for 1 hour, the 9S and 3S peaks disappear, whereas the 4.6S peak is only minimally affected. Therefore, the 9S and 3S peaks represent binding to receptor in the cytosol and nuclear extract.

Although density gradient centrifugation is quite specific in the identification of androgen receptors, inconsistent results have been reported. Steins et al (19) and Nijs et al (21) were unable to demonstrate a

TABLE 2.—Methods utilizing density gradient centrifugation

Author	Ref	Method	n	S value	Remarks
Menon	18	Sucrose density gradient centrifugation (SDGC) with and without charcoal extraction	25–50 fm/g tissue (cytosol)	8–9	8S peak seen in 70% of cytosols.
			100–150 fm/g tissue (nuclear extract)	3	3S peak seen in 90% of 0.4 M KCl nuclear extracts.
Steins	19	SDGC with and without charcoal extraction	—	—	Peak comigratory with TeBG. Steroid specificities identical with TeBG. No receptor seen in 7 samples of benign prostatic hypertrophy.
Hawkins	20	SDGC following charcoal extraction	—	—	3.5S–4.5S peak seen on incubation with ^{3}H-17β-estradiol in 4 of 23 benign prostatic hypertrophy samples. No. 8S peak seen.
Nijs	21	SDGC following charcoal extraction			No peak seen with ^{3}H-DHT in 23 samples of benign prostatic hypertrophy. 8S peak seen in 2 cancers.
Hansson	12	SDGC of void volume of Sephadex G-100 filtration	—	8–8.5	8S–8.5S peak seen. Inhibition with cold competitors not reported. Steroid or tissue specificities (other than serum) not reported (*see* table 1 also).
Rosen	16	Glycerol gradient centrifugation	30–50 fm/g tissue	8	Tissue specificity not reported (*see* table 1 also).
Hsu	22	SDGC following ammonium sulfate fractionation	—	5	5S peak comigratory with TeBG present in 30%–50% fraction. No peak in 0–33% fraction. No 8S peak. Peak inhibited by 17β-estradiol.
Davis	23	SDGC following, in some instances, ammonium sulfate fractionation and Sephadex G-25 desalting	—	8	8S peak seen in 30% cytosol. Combination experiments with rat prostate and spleen successful. Steroid specificities not reported.
Mainwaring	13	SDGC following Sephadex G-25 desalting	—	8≓3, cytosol	8S peak converted reversibly to a 3.5S in 0.5 M KCl seen in 50% benign prostatic hypertrophy cytosol. Peak suppressed with excess DHT and cyproterone acetate, but not cortisol.
				4.5S, nucleus	4.5S peak seen in nuclear extract.

specific androgen receptor in any of their samples of benign prostatic hyperplasia. Davies and Griffiths (23) demonstrated an 8S peak in 30% and Mainwaring and Milroy (13) in 52% of samples analyzed. In our laboratory (18) we have demonstrated the presence of the receptor in 70% of cytosol preparations and in 90% of nuclear extracts. However, in multiple assays of the same tissue, the results were not always reproducible.

Methods Utilizing Ion-Exchange Chromatography (Table 3)

Because the receptor is eluted from DEAE cellulose columns by solutions of high ionic strength (0.4 M) whereas TeBG is eluted by low ionic strength buffers (0.04 M) (22), this method appears highly specific for analyzing the androgen receptor. Extensive purification of the cytosol, however, is required

TABLE 3.—Methods utilizing ion-exchange chromatography

Author	Ref	Method	n	Kd	Remarks
Fang	11	Ammonium sulfate fractionation, Sephadex G-100 filtration, DEAE cellulose chromatography	—	—	9.5S peak that was not seen with serum (*see* also table 1).
Hsu	21	Ammonium sulfate fractionation, dialysis with 0.0175 phosphate buffer, DEAE cellulose chromatography	—	—	TeBG eluted with 0.04 M phosphate; receptor eluted with 0.4 M phosphate (*see* also table 2).

TABLE 4.—Methods utilizing gel electrophoresis

Author	Ref	Method	n	Kd	Remarks
Wagner	24	Agar gel electrophoresis of charcoal-extracted cytosol	27 fm/mg protein	—	Preliminary abstract. Receptor content variable in patients with cancer of the prostate. No receptor seen in rectus muscle. Steroid specificities not given.
Steins	19	Agar gel electrophoresis of cytosol with and without charcoal extraction	—	—	Anodally migrating peak indistinguishable from albumin. However, amount of binding seen in prostate was more than could be accounted for by serum contamination (*see* also table 2).
Hawkins	20	Agar gel electrophoresis of charcoal-extracted cytosol	—	—	On incubation with ^{3}H-17β-estradiol, anodally migrating compound seen which was diminished on treatment with dextran-coated charcoal (DCC). Inhibited by 1000-fold excess DHT. Similarly migrating compound seen in serum not inhibited by DHT lost on DCC treatment, albumin.
Nijs	21	Agar gel electrophoresis of charcoal-extracted cytosol	—	—	No such compound seen in 23 samples of benign prostatic hypertrophy on incubation with ^{3}H-DHT; however, it was seen in 2 cases of cancer of the prostate.
Rosen	16	Polyacrilamide gel electrophoresis	—	—	3 peaks of radioactivity seen on analysis of cytosol; only 2 (albumin and TeBG) seen on analysis of serum.

and consequently this results in some reduction in receptor content.

Electrophoresis (Table 4)

Agar gel electrophoresis has been utilized by Wagner et al (24), Hawkins et al (20), and Steins et al (19). Though Wagner demonstrated a receptor that migrated toward the anode (n = 21 fm/mg of protein), Steins et al and Nijs et al (21) were unable to demonstrate any evidence of a DHT receptor in human prostatic tissue. Using polyacrilamide gel electrophoresis, Rosen et al (16) demonstrated three peaks of radioactivity in cytosol. Two of these peaks were seen in plasma analyzed under similar conditions and represented binding to albumin and TeBG, but the third, small, and reproducible peak was specific for prostatic cytosol and was thought to be due to receptor.

Miscellaneous (Table 5)

Utilizing a protamine sulfate precipitation assay (18), we have demonstrated high-affinity binding (Kd = 1–5 $\times$ 10^{-9} M) present in small concentrations (100–700 fm/g of tissue) in the cytosol and in the nuclear extract (10–60 fm/g of tissue). This binding is destroyed by heating to 45°C for 1 hour, and is not

TABLE 5.—Miscellaneous methods

Author	Ref	Method	n	Kd	Remarks
Menon	18	Protamine sulfate precipitation	100–700 fm/g tissue (cytosol) 10–60 fm/g tissue (nuclear extract)	$1–5 \times 10^{-9}$ M —	Differential precipitation of acidic receptor by protamine sulfate. High nonspecific background to assay. Receptor seen in 42% cytosols, 56% nuclear extracts.
Rosen	16	Equilibrium dialysis	4000 fm/g tissue	9×10^{-10} M	High-affinity binding in prostatic cytosol indistinguishable from TeBG on glycerol gradients and polyacrilamide gel electrophoresis.
Castañeda	25	Antiserum antibodies	—	—	Work mainly on rat prostate. Brief mention about human tissue experiments given but no details.
Bonne	26	Charcoal assay using synthetic androgen, ^{3}H-methandrostenolone (R1881)	120 fm/g tissue	—	This potent androgen does not bind to TeBG. Work primarily with rat ventral prostate. Preliminary data with human prostate encouraging. Steroid and tissue specificities not reported.

seen with cytosol prepared from rectus muscle, kidney, adult foreskin, fat, or 10% serum. Although the assay is linear with protein concentration, there does not appear to be quantitative precipitation of the receptor. When sucrose density gradient centrifugations were performed before and after extraction of the cytosol with protamine, most but not all of the 9S peak of radioactivity was obliterated. This suggests that although the protamine precipitation assay is specific for the measurement of androgen receptors, it underestimates the concentration of measurable receptor. Utilizing this assay, high-affinity binding was demonstrated in 42% of cytosol preparations and in 56% of nuclear extracts. The relative lack of sensitivity of this assay may be secondary to the high nonspecific background of this technique. Presumably, this may interfere with the measurement of the receptor in small concentrations.

Preliminary results have been reported with three other techniques. Utilizing equilibrium dialysis, Rosen et al (16) demonstrated high-affinity binding in prostatic cytosol that was indistinguishable from binding to TeBG on subsequent glycerol gradient centrifugation or polyacrilamide gel electrophoresis. Castañeda and Liao (25) have suggested the use of antisteroid antibodies to differentiate binding to TeBG from binding to the receptor. However, clinical studies utilizing this assay are lacking. Recently, Bonne and Raynaud (26) have reported on the use of methyltrieneolone, a synthetic androgen that binds with high affinity to the receptor but not to TeBG. This novel steroid may provide a method for distinguishing specific binding to the receptor. A similar approach has been utilized for the identification of several other steroid receptors in man, eg, dexamethasone for the measurement of the cortisol receptor and R5020 for the measurement of the progesterone receptor.

CONCLUSIONS

The measurement of androgen receptors in human prostatic tissue has been plagued with difficulties. One of the major obstacles has been tissue contamination with a serum protein, TeBG, that is present in high concentration and that binds with high affinity to DHT. A second problem has been the high endogenous content of DHT that is present in human prostatic tissue (20 nM). Although it has been estimated that the total receptor content of human prostatic tissue is 2600 fm/g of tissue (16), most of this receptor is bound to endogenous DHT. Because the steroid receptor complex has a long half-life at low temperatures, and because at high temperatures the receptor is destroyed and there is significant metabolism of DHT, there are severe limitations to the use of an exchange assay to estimate total androgen receptor content. Third, the prostate contains large amounts of proteolytic enzymes. These three factors may be responsible for some of the conflicting data that we have summarized in this review of the literature.

From the data presented in this paper, it is clear that sucrose density gradient centrifugation, ion-exchange chromatography, and the protamine precipitation assay have provided specific means for the identification of androgen receptors in human prostatic tissue. The binding measured by these techniques is high affinity, tissue specific, and ste-

roid specific. In addition, the binding measured by these assays differs from binding to TeBG in several important ways: sedimentation coefficient, heat lability, charge, and protamine precipitability. Consequently, the measurement of androgen receptors utilizing these techniques appears to be unaffected by tissue contamination with serum proteins. Unfortunately, these techniques are either cumbersome or lack the reproducibility that is necessary for an assay that is to have clinical applicability.

We hope that in the near future a reliable assay will be developed that can be sufficiently miniaturized to enable the measurement of androgen receptors on tissue specimens obtained at the time of needle biopsy. This technique may provide important insight into the biologic behavior of prostatic carcinoma. If the hormonal responsiveness of prostatic cancer can be correlated with the presence of cytoplasmic receptor proteins, this information may be valuable in assigning therapy. For example, the correlation of receptor content and hormonal responsiveness may help to identify the select subpopulation of patients with hormone-resistant cancers. If this were possible, the feminizing side effects of castration and estrogen therapy could be avoided in these patients and their tumor could be attacked more aggressively with other modalities of treatment such as surgery, radiation therapy, or chemotherapy. In patients who have exacerbation of symptoms following initial hormonal therapy, it may be possible to select the few who will benefit from adrenalectomy or hypophysectomy. The measurement of androgen receptors may give further insight into the biologic behavior of prostatic carcinomas and provide a method for assigning definitive therapy in individual patients on a more rational basis.

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Characterization of the Dunning R3327H Prostatic Adenocarcinoma: An Appropriate Animal Model for Prostatic Cancer [1]

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SUMMARY

The Dunning R3327H rat prostatic adenocarcinoma appears to be an appropriate animal model for studying prostatic cancer. This report contains a detailed characterization of this tumor at the morphologic, biochemical, and therapeutic levels. Electron micrographic, histologic, and histochemical studies clearly establish the adenocarcinoma nature of this tumor. The histology of the R3327H tumor is similar to well-differentiated human prostatic cancer. The biochemical and enzymatic profile of the tumor indicates its origin from the rat dorsolateral prostate. The cell kinetics and growth rates of this tumor following a variety of hormonal manipulations (castration, estrogens, androgens, and antiandrogens) have established that 70%–90% of the cells in this tumor require androgens for their growth. However, 10%–30% of the cells are capable of growth in the absence of androgens. Both cell types are present in the initial tumor inoculum and these different cell types possess similar growth rates. The predominance of the androgen-sensitive cells accounts for the relatively greater size of the tumor achieved in the intact male animal at a given growth time. After the tumor is well established in an intact animal, subsequent estrogen therapy or castration resulted in a marked diminution in tumor volume. This was followed by a subsequent relapse. In addition, estramustine phosphate was also shown to cause shrinkage in the tumor volume.

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ANIMAL MODEL

The study of potential non-hormonal cancer chemotherapeutic agents for human prostatic adenocarcinoma has been hampered for several reasons. The natural history of the disease is variable and unpredictable, with lack of agreement on when and what treatment must be given at the different stages of disease. The often dramatic clinical response to hormonal manipulation has led to a reliance on this mode of therapy for symptomatic advanced prostatic cancer, but this is almost invariably followed by relapse. Well-controlled, randomized trials of non-hormonal cytotoxic chemotherapeutic agents in prostatic cancer have been limited (1–5). The final impediment to progress in this area has been the absence of an appropriate animal model for prostatic adenocarcinoma.

The ideal criteria for an animal model of prostatic carcinoma would include the spontaneous appearance, in a aged animal, of a prostatic adenocarcinoma histologically and biochemically similar to human prostatic cancer. Furthermore, this animal tumor should metastasize, be androgen dependent, and show a response to castration and estrogens with subsequent relapse. Finally, the tumor should

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TABLE 1.—Spontaneous prostatic adenocarcinomas in animals

Species	Yr	Investigators	Tumor
Rat	1961	W.F. Dunning	Dorsal prostate; Copenhagen rat, aged, syngeneic; androgen dependent; transplantable
	1973	M. Pollard P.H. Luckert	Aged, germ-free Lobund Wistar; ? lobe, prostate; hormone sensitive; transplantable
	1975	S.A. Shain B. McCullough A. Segaloff	Aged A × C rats; ventral prostate; no metastases; not transplanted
Hamster	1960	J.G. Fortner J.W. Funkhauser M.R. Cullen	Aged Syrian golden hamster; transplantable; lost
Dog	1968	I. Leav G.V. Ling	Aged (> 8 yrs) mongrels; metastases; no occult tumor
Mastomy	1965	K.C. Snell H.L. Stewart	Aged female African rodent prostate
	1970	J. Holland	
Monkey	1940	E.T. Engel A.P. Stout	Aged *Macaca mulatta*

be transplantable into large numbers of syngeneic animals and have characteristics which provide parameters which can be monitored for diagnostic and therapeutic studies. The ultimate animal model should provide accurate predictions for human therapy.

Table 1 lists the animal species in which spontaneous adenocarcinoma of the prostate have been reported (6–12). This report describes our experience with the Dunning R3327H prostatic adenocarcinoma in the rat.

History of the Dunning R3327 Tumor

In August 1961, Dr. W. F. Dunning of the University of Florida observed a spontaneous tumor of the prostate which occurred at necropsy in a Copenhagen male rat from the 54th brother × sister generation of line 2331. The rat was a 22-month-old retired breeder and the tumor occupied a large portion of the lower abdominal cavity and appeared to involve primarily the dorsal prostate gland. This primary tumor was classified as a papillary adenocarcinoma and no metastases were identified. Tenmg grafts of the soft tumor tissue were transplanted to ten rats, four of the same inbred line as the host of the primary tumor and six F_1 hybrids from a Copenhagen × Fischer cross. The transplanted tumors grew very slowly and became palpable on the 60th day. Histologic analysis of this transplanted tumor on the 245th day indicated glandular formation and cellular material corresponding to a normal dorsal-type gland of the rat prostate. The tumor was positive to periodic acid-Schiff stain and negative to the dithizone stain typical of dorsal-type glands. Samples of heart blood from the rat did not indicate an elevated serum-acid phosphatase level. These findings were reported by Dr. Dunning in 1963 (13).

This tumor remains histologically a well-differentiated adenocarcinoma. In 1974, Voigt and Dunning reported on the hormone sensitivity and metabolism of testosterone in the tumor line (14). In an in vivo study, they were able to demonstrate the 5α-reduced androgen metabolites in this tumor line (15). In addition, they observed differences in the growth rate between males and castrates, thus establishing the hormone dependency of the tumor.

MATERIALS AND METHODS

Animals

Rats used in these experiments were of two types: an inbred Copenhagen strain and the first generation (F_1) hybrid of the mating of a Copenhagen male and a Fischer female. Comparative studies have shown that tumors in either strain are comparable histologically and biochemically and in their growth patterns.

The animals were housed in cages containing three to five animals. One male serviced three to four females. Using these methods, healthy litters,

with a survivorship $\geq$ 90% were obtained, once the good breeding mothers had been selected.

Tumor

The original source of our tumor was from a Copenhagen male rat bearing a subcutaneous tumor injected at the Mason Research Institute. Once the tumor had reached a size of 2 cm in diameter, it was excised, minced with scissors, passed through two wire mesh gauze strainers (1.5 and 0.5 mm), and resuspended in cold RPMI 1640 cell culture medium. Cell suspensions were then made up to a dose of 10^6–10^7 viable cells/ml as determined by trypan blue exclusion. The usual cell suspension contained 25% viable cells. One ml of the resultant cell suspension was then injected sc into the flank of the recipient animal.

The tumor used in our studies was a homogenous, glistening white solid tumor which histologically proved to be a relatively pure, well-differentiated adenocarcinoma. The term tumor in this report refers to the R3327H tumor unless otherwise indicated. This new code was chosen to define the specific line which we have characterized in this report.

Growth Rates

Following the sc injection of tumor cell suspension, tumors were measured in all animals every 2–4 weeks with micro-calipers. Three diameters of the mass were determined, and the tumor volume was calculated according to the formula [l × w × h × 0.5236] described by Janik et al (16). When the tumor was excised the actual volume and tumor weight were measured. Tumor growth rate was further characterized by injecting 200 microcuries of tritiated thymidine (58 curies/mmol) 3 hours prior to tumor harvesting and then determining the rate of DNA synthesis as reflected in thymidine incorporation. Total tissue DNA, RNA, and protein were measured according to the method described by Coffey, Shimazaki, and Williams-Ashman (17). Using the tumor volume, weight, and total DNA per mg of tissue, as well as the DNA content per isolated nucleus, it was possible to determine the total number of cells per tumor.

The measured tumor volumes along the growth curve of each tumor were analyzed semi-logarithmically and computerized extrapolations were made to zero time at original injection to estimate the apparent number of tumor cells viable at the time of original cell inoculation. Tumor doubling times were measured on this same curve. The slope of the semi-log plot (K), determined by statistical linear regression, determined the actual growth rate of each tumor. At time of death or sacrifice, complete autopsies were performed and areas suggesting metastasis were histologically analyzed.

Morphologic Determination

Tumor biopsies, all normal sex accessory tissues, and the control tissues of liver and kidney were routinely stained with hematoxylin and eosin, periodic acid-Schiff reagent, and Masson trichrome stain. Enzymatic histochemical staining was done on frozen sections for the 20 enzymes listed in table 2. Electron microscopic studies were performed on tissue sections which were fixed in 4% glutaraldehyde and washed in 0.1 M cacodylate buffer, pH 7.4. Post-fixation was done in 1% osmium tetroxide, imbedded in a mixture of epon and araldite, stained with uranyl acetate and lead citrate, and viewed in a Hitachi HU11F electron microscope.

Biochemical Determination

Measurements on the tumor, rat sex accessory tissues (dorsolateral and ventral prostate, coagulating gland, and seminal vesicle), as well as control tissues (liver, kidney, lung, diaphragm), were made for chemical and enzymatic determinants. Animals were sacrificed by ether anesthesia or decapitation. Tissues were rapidly removed, weighed, chilled, and homogenized on the Polytron tissue homogenizer. For the chemical markers citric acid (18) and fructose (19), the tissues were homogenized in perchloric acid. For enzymatic determination, homogenization was in 0.25 M sucrose and 0.5 mM phosphate buffer. The following enzymes were measured: acid phosphatase (20), alkaline phosphatase (21), β-glucuronidase (22), leucine aminopeptidase (23), γ-glutamyl transpeptidase (24), and 5α-reductase (25).

Therapeutic Agents

Experiments were conducted on groups of animals which had been randomized on the basis of tumor volume, in order to determine the effect of hormonal and non-hormonal chemotherapeutic agents on the growth of the recently injected as well as the established tumor.

The following agents were used: (*a*) testosterone propionate (Sigma Chemical Co, St. Louis, Mo); (*b*) diethylstilbestrol (DES) (E. R. Squibb and Sons, New Brunswick, NJ); (*c*) flutamide (Schering Corporation, Bloomfield, NJ; Sch 13521); (*d*) estramustine phosphate (AB Leo, Helsingborg, Sweden, Estracyt); (*e*) sesame oil; and (*f*) 0.9% saline.

TABLE 2.—Histochemical properties of the rat prostate epithelium and R3327H adenocarcinoma*

Enzyme†	Prostate			R3327H adenocarcinoma
	Dorsal	Lateral	Ventral	
Acid phosphatase	+++	++	+++	+++
Alkaline phosphatase	+	+	++	++
Acetylcholinesterase	–	–	–	+
5'-Nucleotidase	–	++	±+	+++
ATPase	+	+	+	+++
α-Glycerol phosphate DH	+++++	++++	+++++	+++
β-OH butyric DH	+++	++++	++	+++
Butyryl cholinesterase	–	–	–	++
Cytochrome oxidase	+	+/–	+	++
NAD diaphorase	+++++	++++	+++++	++++
Glucose-6-phosphate DH	++	+++	+++	++
β-Glucuronidase	++	+/–	+	++
Isopropyl alcohol DH	+	+	+++	++
Leucine aminopeptidase	+	++++	++	+
Monoamine oxidase	–	+/–	–	+++
Propyl alcohol DH	+++	+	++	++
Nonspecific esterase	++++++	+++++	+++++	++++
NADP diaphorase	++	+++	++++	++
Isocitric acid DH	+	++	+++	++
Succinic acid DH	+++	+++++	+++++	++++

*– = negative; + = positive.
†DH = dehydrogenase.

Dosages for injection of these agents were based on m^2 determination as reported by Freireich and others (26). For the non-hormonal chemotherapeutic agents, dose, route, and schedule of injection were chosen to closely approach those used by the National Prostatic Cancer Project in their Clinical Protocols 100 and 200 (6).

RESULTS

Tumor Viability

The tumor was transplanted at a dose of 10^6–10^7 cells into 170 male rats and 160 of these grew tumors (94%). Dose-response curves of cell inoculation indicated that when $< 10^4$ viable cells were injected tumor grew in < 50% of the male rats. In intact males, an injection of 10^6–10^7 cells resulted in a tumor with a diameter of 1–2 cm after about 100 days of growth. In castrate males and females, the tumor reached this size only after 6–7 months of growth in 19 of 24 (78%) of the animals injected. The characteristics of the tumor which grows in the animal who lacks androgen will be discussed in the section below entitled *Hormonal Requirements of Tumor Growth.*

Pathology

Histologically, the R3327H tumor is a moderately to well-differentiated adenocarcinoma with acinar formation, cellular and nuclear pleomorphism, and little interacinar stroma (fig 1A). There is a similarity to the histology found in a well-differentiated human prostatic adenocarcinoma (fig 1B). Both tumors have the pattern of one layer of epithelial cells per acinus which Mostofi emphasizes as characteristic of prostatic carcinoma (27). Electron micrographs of this tumor demonstrate both the nuclear pleomorphism and the microvilli which confirm the epithelial characteristics of these cells (fig 2).

The tumor is marked by encapsulation, although a few tumors have invaded surrounding muscle, as well as erosion into the peritoneal cavity. After apparently adequate surgical excision of the tumor, it will regrow at the original site in 75% of the animals. Metastases to the retroperitoneal lymph nodes and lungs have been demonstrated (figs 3A and 3B). The tumor has a low incidence of metastases (5%) but this can be enhanced by hormonal manipulation.

Biochemical Profile

The biochemical indices measured have determined that this tumor model closely resembles the dorsolateral prostate of the Copenhagen rat. Table 3 lists five enzymes which have been determined in the tumor, as well as the listed tissues as controls. By setting the enzymatic activity (nmols/mg protein/10 minutes) within the tumor tissue relative to 1.0 and comparing the relative activity of the same enzymes within the control tissues, the computerized correlation coefficients (0.96) clearly indicate a

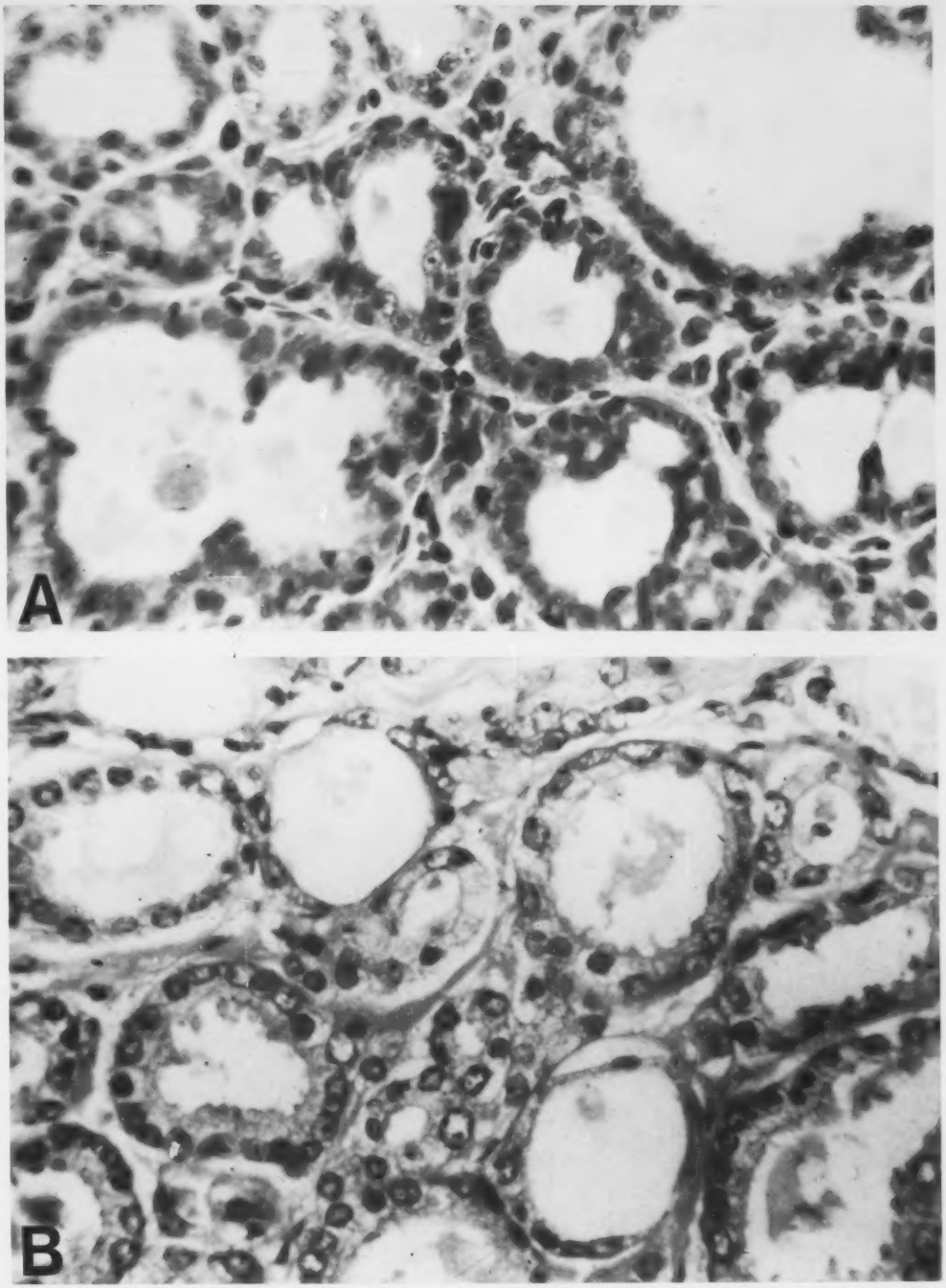

FIGURE 1.—(A) Histology of the R3327H tumor (hematoxylin and eosin stain). (B) Well-differentiated human prostatic adenocarcinoma.

TABLE 3.—Relative enzymatic activity in various rat tissues

Enzymes	Tissue						
	R3327H tumor*	Dorsolateral prostate	Ventral prostate	Coagulating gland	Seminal vesicle	Kidney	Liver
Aminopeptidase	1.0	0.67	4.1	0.73	0.2	6.9	0.33
γ-Glutamyl transpeptidase	1.0	0.50	0.13	0.26	3.1	94	0.07
Acid phosphatase	1.0	0.51	0.91	0.57	0.41	1.1	1.1
Alkaline phosphatase	1.0	0.62	2.5	0.74	0.77	1.2	0.03
β-Glucuronidase	1.0	0.36	0.5	0.03	0.14	0.11	1.4
Correlation coefficient	1.0	0.96	0.44	0.52	0.36	0.36	0.42

*Relative activity of 1.0 for tumor is equivalent to the following (nmols/mg total protein/10 mins): aminopeptidase, 10.6 ± 2.0; γ-glutamyl transpeptidase, 5.0 ± 0.2; acid phosphatase, 5.7 ± 0.1; alkaline phosphatase 96.0 ± 16; β-glucuronidase, 4.6 ± 1.0. 6 individual tumors for each value in triplicate.

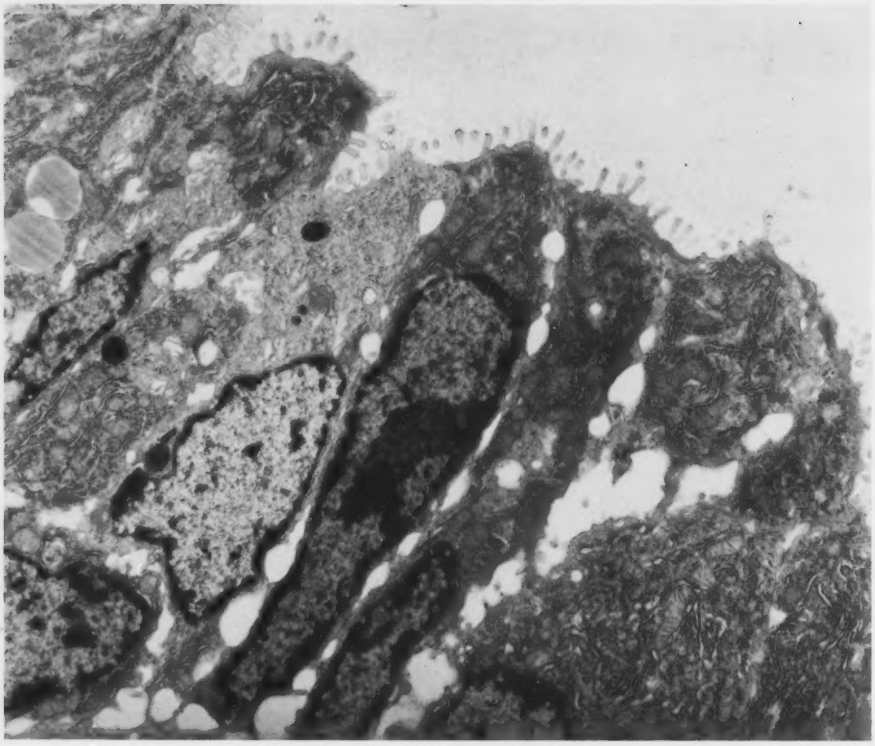

FIGURE 2.—Electron micrograph, R3327H tumor.

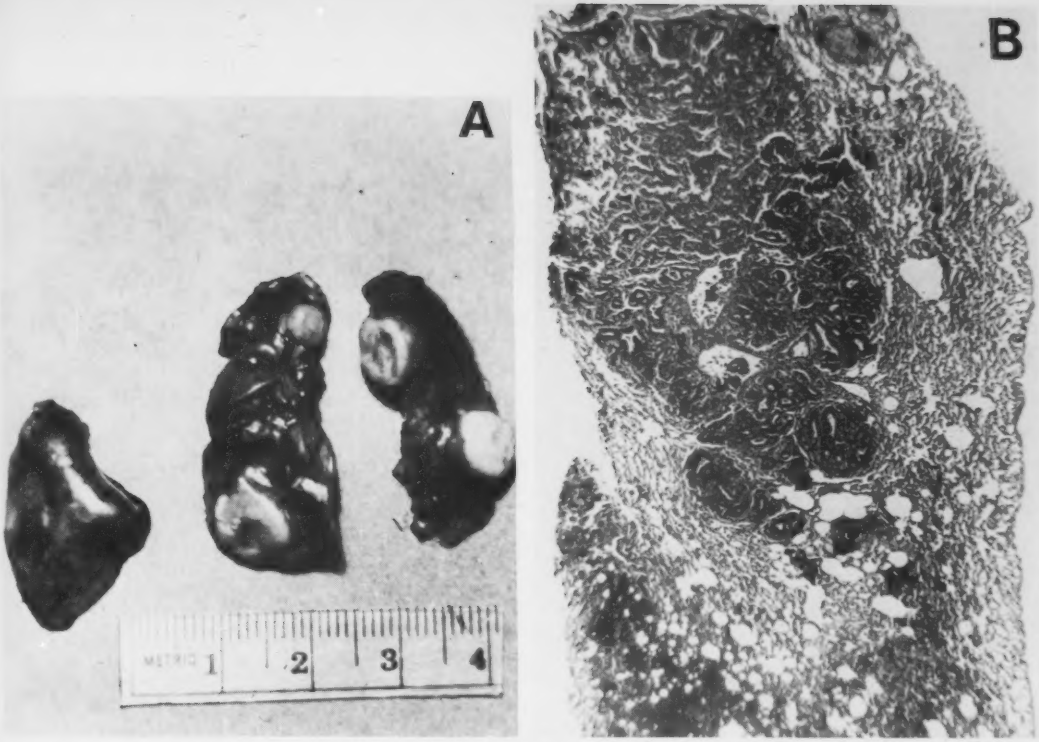

FIGURE 3.—(A) Gross appearance, pulmonary metastases of the R3327H tumor. (B) Histologic appearance (hematoxylin and eosin stain) of the R3327H tumor.

close enzymatic relationship between the R3327H tumor and the dorsolateral prostate of the rat.

Lower levels of citric acid (5 ± 1 μg/100 g tissue wet weight) and fructose (11 ± 1) were observed in the rat tumor when compared to the levels in normal dorsolateral prostate (citric 25 ± 5; fructose 140 ± 25). A similar diminution in citric acid is observed between normal and prostatic adenocarcinoma in the human (28).

Histochemical Profile

Twenty different histochemical stains have been performed on the tumor, and the rat's ventral, lateral, and dorsal prostate. The amount of stain was graded by a histochemist and assigned a value of from 0 to 6+ depending on the intensity of the reaction in each tissue. By comparing the relative activity of the tumor with that in each of the prostatic lobes, it is apparent that the epithelium of the tumor has a correlation with the epithelium of the lobes of the normal rat prostate gland. As opposed to the biochemical assays, no specific lobe of origin could be identified with these histochemical methods.

Hormonal Requirements of the Tumor

Figure 4 depicts the linear and the logarithmic growth plot of the R3327H tumor under a variety of hormone conditions. It is evident that the R3327H tumor achieves a comparatively greater size in the presence of androgen.

Extrapolating the semi-log growth curves back to zero time indicates the initial volumes of cells in the inoculum responding to each hormonal manipulation. Each group was initially injected with identical 1.5×10^6 cells of the same tumor suspension. Determining the number of cells per mm^3 following each hormonal condition and knowing the extrapolated volume, it is possible to determine the percent of the total of the 1.5×10^6 cells present initially which responded in each hormone condition. This is presented in figure 5 and indicates that 93% of the

TABLE 4.—Growth parameters of the R3327H tumor

Hormonal status	Tumor doubling time, volume (days)	Growth rate,† slope, log vol/day ($\times 10^3$)	Initial tumor‡ (at Day 0)				Final tumor (at Day 180)			
			Extrapolated initial volume§		Apparent cells responding¶		Tumor wet weight (mg)	Tumor volume (mm^3)	Total cells** (10^6)	Cells/mm^3 (10^6)
			(log mm^3)	(mm^3)	No. (10^6)	%				
Intact	20.9	33	1.24	17.4	1.38	93	3475 ± 1485	7857 ± 2789	6.48 ± 2.7	0.82
Intact + testosterone propionate	18.7	37	1.19	15.3	1.10	74	4255 ± 1089	13,199 ± 3896	6.40 ± 1.7	0.48
Castrate + testosterone propionate	19.8	35	1.14	14.0	1.24	83	5257 ± 1695	11,140 ± 4060	8.73 ± 3.0	0.78
Intact + flutamide	20.3	34	0.24	1.64	0.25	16	597 ± 190	962 ± 342	1.20 ± 0.32	1.25
Intact + DES	20.2	39	0.007	0.98	0.13	8	407 ± 65	739 ± 273	0.65 ± 0.21	0.87
Castrate	24.8	27	0.47	2.96	0.44	29	307 ± 75	474 ± 97	0.68 ± 0.16	1.40

*Doubling time $= \dfrac{\text{(days of growth = 180)}}{\log \text{(final volume)} - \log \text{(initial volume)}}$

†Slope $= \dfrac{\log 2}{\dfrac{[\log \text{(final volume)} = \log \text{(initial volume)}]\ 1n\ 2}{180}}$

‡Initial inoculum at Day 0: 1.5×10^6 viable cells.

§Initial volume: statistical linear regression from semi-log growth curve to volume intercept at 0 days.

¶Apparent cells responding of the initial inoculum $= \dfrac{\text{(initial volume) (cells/mm}^3\text{) (100)}}{1.5 \times 10^6}$.

**Total cells $= \dfrac{\text{(total DNA)}}{\text{(DNA/nucleus)}}$.

injected cells are capable of growth in the intact animal. Likewise, 83% of the total cell injected were capable of growth in castrates receiving exogenous testosterone. However, in androgen-deprived animals, such as the castrates or intacts treated with estrogen, the growing cells represent only 8%–29%. This is interpreted to mean that the original inoculum contained both hormonally sensitive and insensitive cells with the former predominating.

From the slopes of the semi-log growth curve (fig 4B), one can determine the growth rates and doubling times of the tumor (table 4). It is apparent that the doubling times of both the hormone sensitive and insensitive cells are essentially similar.

From the above considerations, it is evident that the difference in the size of the tumor at similar points in time is accounted for by the fact that apparently different numbers of tumor cells were initially able to begin growth in the different hormonal environments. Since hormonally sensitive and insensitive cells are both present in the initial inoculum, one would expect a different histologic appearance of the tumor which grows out in intact and castrate animals. The tumor present in the castrate host is depicted in figure 6 and should be compared to figure 1A which was obtained in an intact host.

The above studies indicated the hormone requirements of this tumor during initial stages of tumor growth. Clinically, one deals with an established tumor and provides subsequent therapy by hormonal manipulation. To approach this problem, we permitted the tumor to grow for 120 days in intact animals and to reach a volume of approximately 30 cm^3. At 120 days, the animals were then randomized by tumor size and treated as controls, with castration, or with DES. The individual responses to 14 days of therapy (120–134 days) are depicted in figure 7. All of the control tumors increased in size by an overall average of 117%. Seventy-two percent of the animals responded to castration or DES therapy. The castrate animals had an average decrease of 36% of tumor volume, with DES increasing an average of 3%, although individual responses were noted. This pattern of response is similar to what is observed in human prostatic adenocarcinoma.

After the initial response to castration, the tumor relapses (fig 8). The ultimate relapse of most hormonally responsive prostatic adenocarcinomas is from a clone (or clones) of hormonally unresponsive

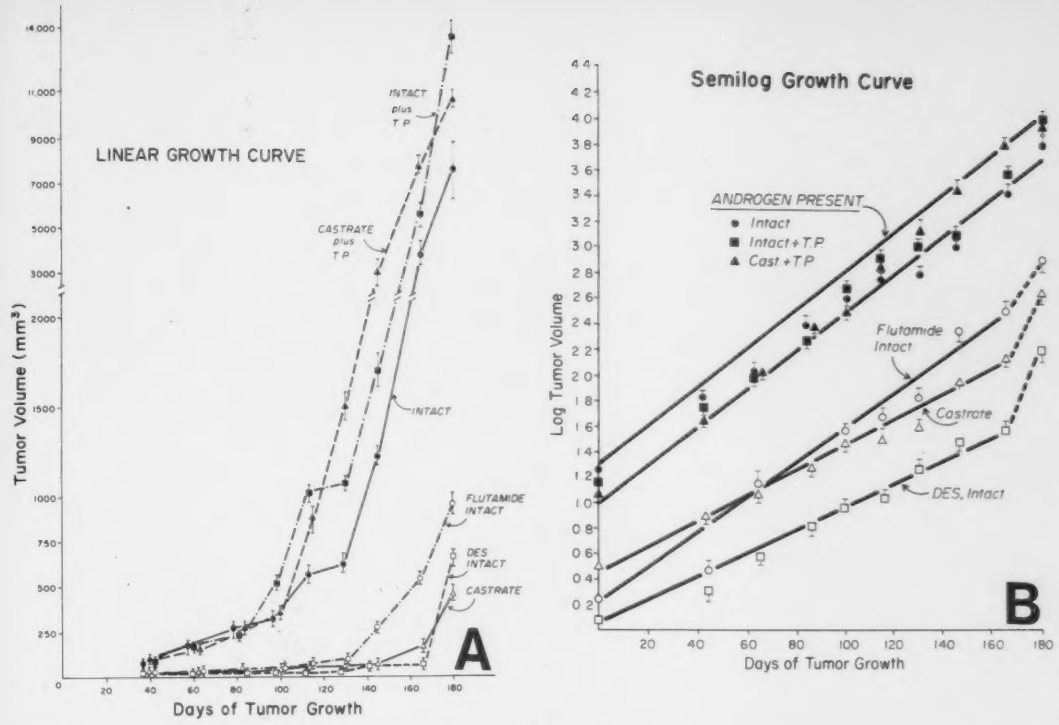

FIGURE 4.—Linear (A) and semilogarithmic (B) growth plot of the R3327H tumor under various hormone conditions for 180 days (10 animals/group): (1) intact animal, sesame oil, 2 ml/day sc; (2) intact + testosterone propionate (TP), 2 mg/day; (3) castrate + TP, 2 mg/day; (4) intact + DES, 100 μg/kg/day; (5) intact + flutamide, 50 mg/kg/day; (6) castrate, sesame oil. Initial inoculum at Day 0, 1.5×10^6 cells.

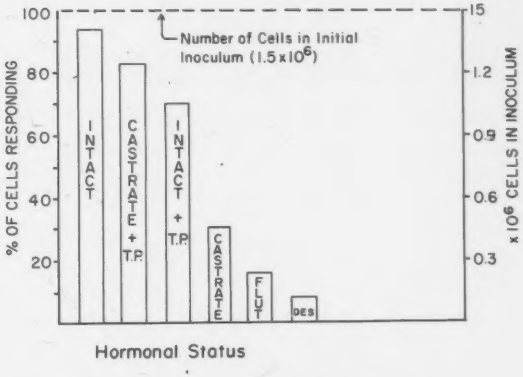

FIGURE 5.—% of cells responding to each hormonal manipulation.

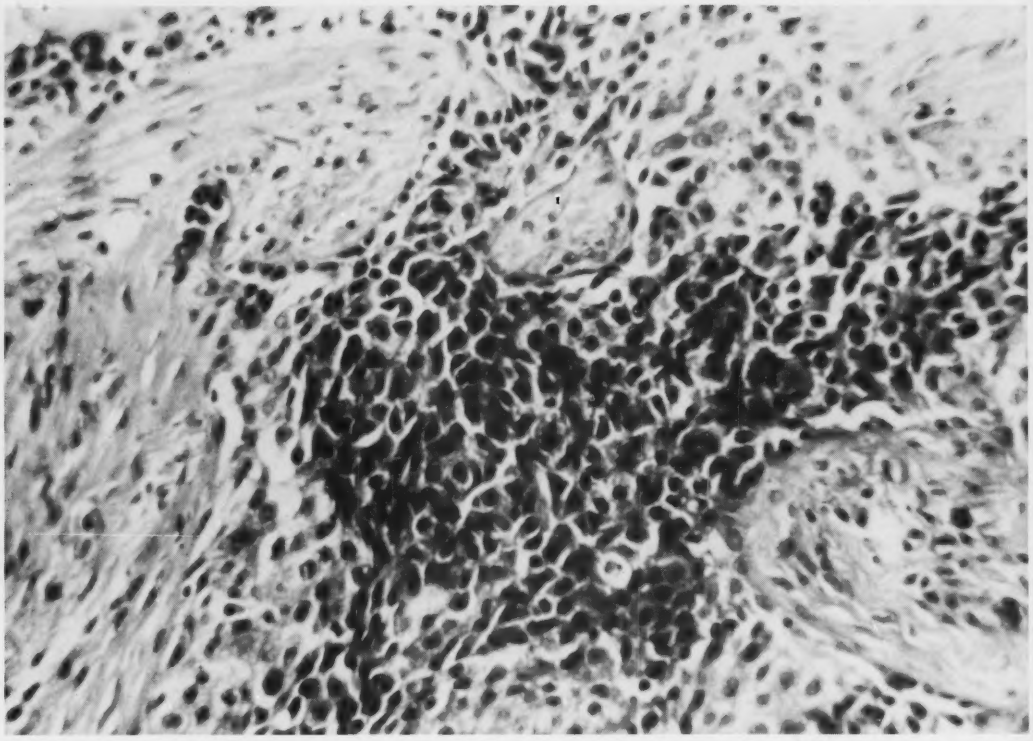

FIGURE 6.—Histologic appearance of the tumor which grew in the castrate rat (hematoxylin and eosin stain), 180 days.

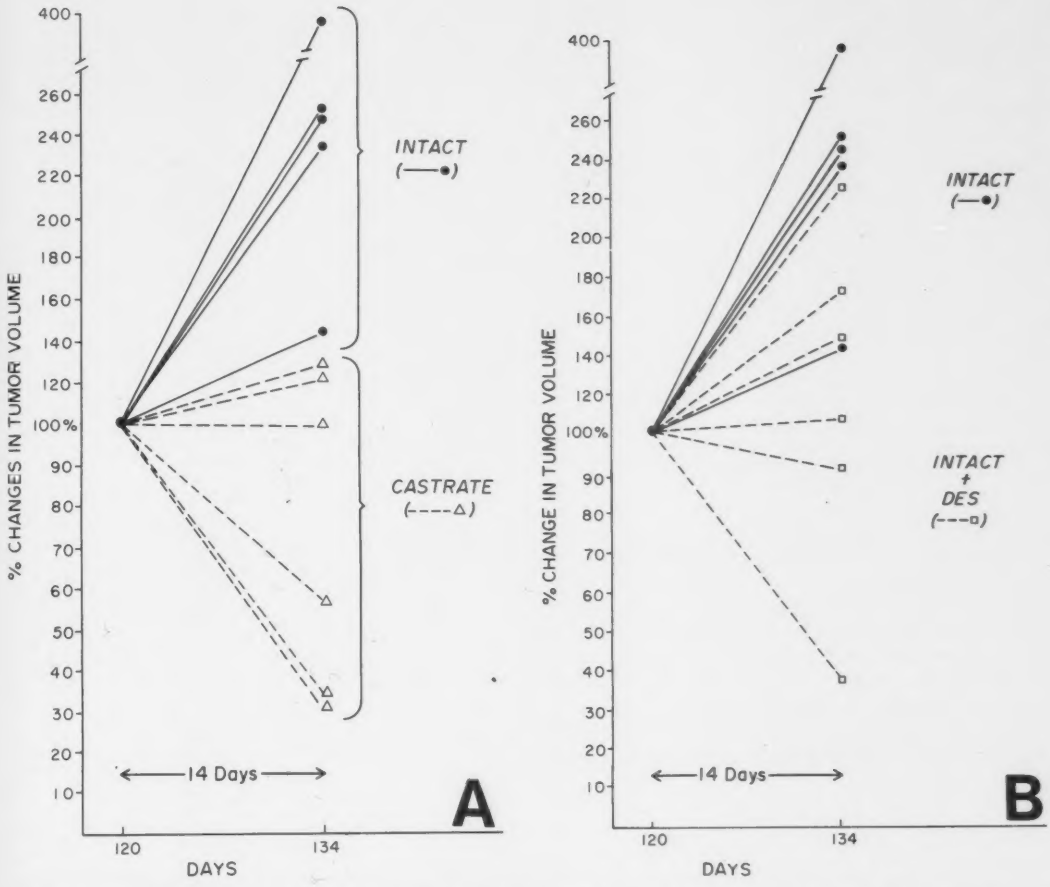

FIGURE 7.—(A) Individual response of the established R3327H tumor to castration after 120 days of growth in an intact animal. Animals were castrated on Day 120. 100% = 33.2 ± 7.1 cm^3 tumor volume. (B) Individual response of the established R3327H tumor to DES, 100 μg/kg/day sc. Animals were treated with estrogen on Days 120–134. 100% = 32.9 ± 11.0 cm^3 tumor volume at 120 days in intact animals.

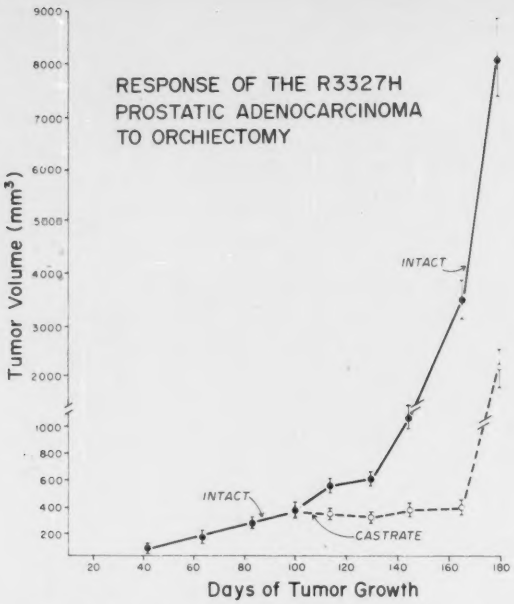

FIGURE 8.—Relapse of the R3327H tumor following initial response to castration.

cells within the tumor, present from early phases of tumor growth. Non-hormonal chemotherapy, therefore, needs to be evaluated for its effectiveness in all stages of this disease. We have performed preliminary experiments utilizing past, present, and future protocols of the National Prostatic Cancer Project as guidelines, employing non-hormonal chemotherapy in this tumor model system.

Figure 9 presents the response of the established R3327H tumor to daily ip injections of estramustine phosphate at a dose of 600 mg/m²/day for 14 days of therapy. The tumor in these animals lost an average volume of 20%, compared with an increase in volume of 117% in the intact control group which received ip injections of saline daily.

Other Dunning Prostatic Tumor Lines

During the 4th generation of tumor transfer at our facility, a rapidly growing tumor arose following the usual tumor transplant procedures. This has been cloned out to be an anaplastic tumor which grew to a size equal to 50% of the rat's body weight in 30 days and has a doubling time of 2 days. Furthermore, it grew equally well in males, females, and castrate males. Histologically, biochemically, and histochemically this anaplastic variety does not resemble either the hormonally dependent R3327H

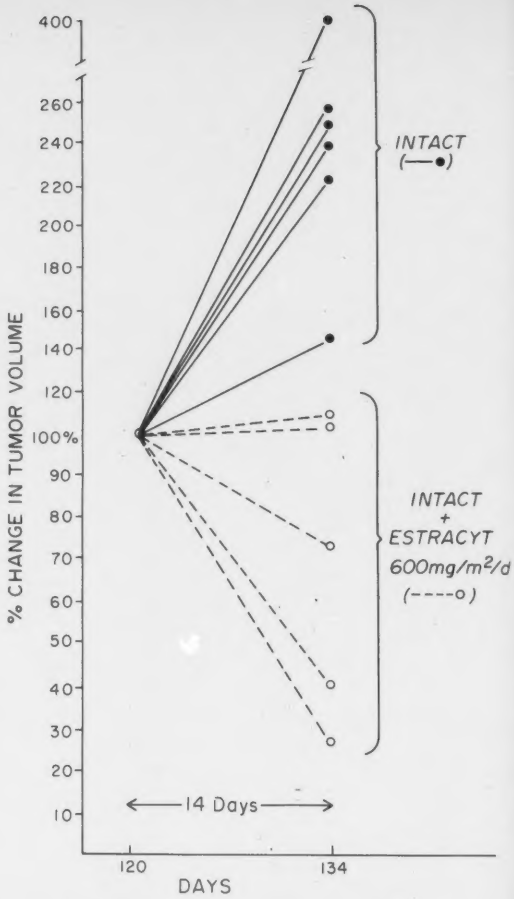

FIGURE 9.—Response of the established R3327H tumor to estramustine phosphate (Estracyt), 600 mg/m²/day ip after 120 days of growth, 14 days of therapy. Intact animals.

adenocarcinoma or the normal rat prostatic tissue (fig 10).

Voigt et al have also reported a non-hormonally dependent tumor (R3327A) which arose during the 5th tumor generation in 1965 (15). Their variety proved to be a squamous cell carcinoma which lacked both the 5α-reductase enzyme as well as DHT cytoplasmic receptor proteins. Because of the various histologic types of tumors which have arisen from the original tumor source, it is essential to define each tumor so that future studies are comparable. Table 5 lists these currently available tumors which have arisen from the original Dunning R3327 prostatic adenocarcinoma line. Histologic differentiation, hormonal sensitivity, and growth rate have been used as the basic point among which to distin-

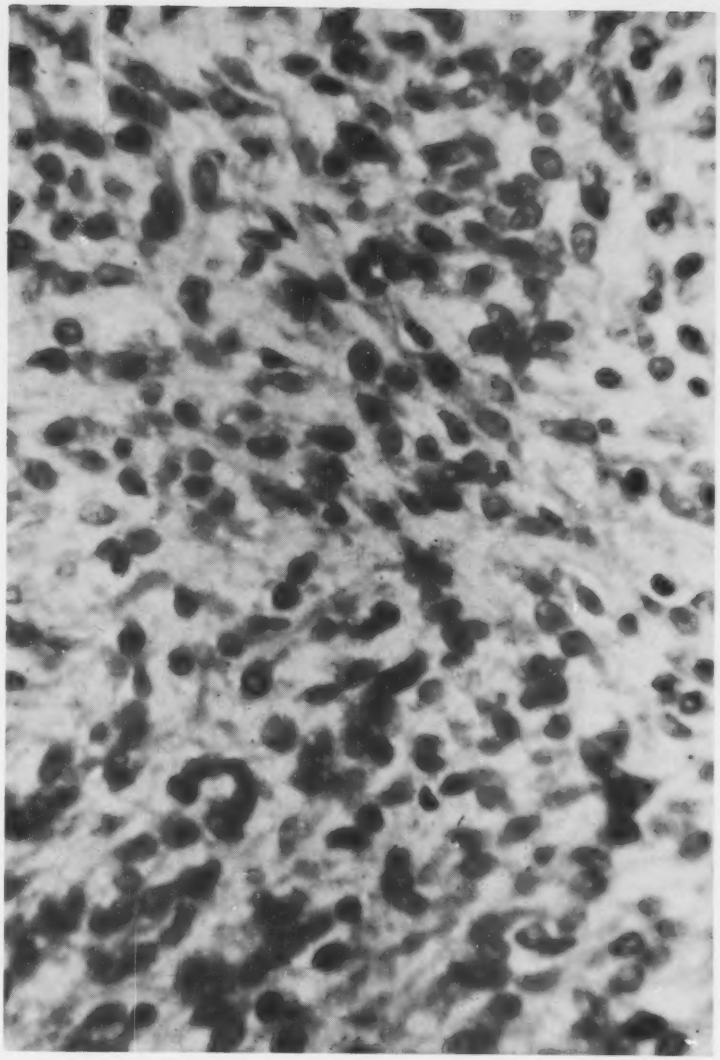

FIGURE 10.—Histology of the R3327 AT anaplastic tumor.

TABLE 5.—Nomenclature of the Dunning R3327 prostatic carcinoma

Nomenclature	Histologic type	Comment
R3327 (R3327C)	Adenocarcinoma	Original Dunning tumor; androgen dependent; osteometaplasia within adenocarcinoma
R3327A	Squamous	Non-hormonally dependent; large fluid-filled cysts
R3327H	Adenocarcinoma	Androgen dependent; moderately to well-differentiated with little interacinar stroma and no bone formation
R3327AT	Anaplastic	Very rapidly growing, non-hormonally dependent tumor; no cystic formation or necrosis; biochemically not related to rat prostatic lobes

guish each tumor. The experiments reported in this paper were performed on the R3327H tumor.

DISCUSSION

The R3327H prostatic adenocarcinoma fulfills the criteria for an animal model of carcinoma of the prostate. The original tumor arose spontaneously in an aged male rat; it grows slowly, demonstrates malignant cellular characteristics, and metastasizes. The androgen dependence of most of the cells within this tumor mimics the human situation as does the occurrence of a non-hormonally dependent phase of tumor growth. The morphologic and biochemical profile prove that it closely resembles its origin from the dorsolateral prostate of the rat, as originally suggested by Dunning. The tumor can be predictably grown in large numbers of syngeneic animals thereby providing sufficient numbers for studying this tumor.

Although several subvarieties of tumor have arisen from the original source, a well-differentiated, androgen-dependent adenocarcinoma can be transplanted if the tumor is harvested when it is 2–3 cm in diameter, and its gross appearance resembles that which we have described, that is, white, glistening, and homogenous. The other tumor varieties are of interest for the comparative studies, as well as to provide clues into their cell source of origin.

The hormonal dependency studies which we have presented clearly indicate that several cell types exist in the inoculated tumor state. From 70% to 90% of the cells in the R3327H tumor appear to be dependent on androgen for their growth stimulation. However, the tumor also contains between 8% and 30% of the cells which do not require androgen. These cells form tumors in this system which grow at a rate almost equal to the androgen-dependent tumor. The fewer number of original cells accounts for the comparative size difference in these tumors at similar intervals. In the later phase of tumor growth, it is likely that either the original androgen-dependent clones of cells lose this reliance on the hormone, or that the originally androgen-independent clones predominate. This can account for the period of relapse seen following an original response to castration or DES.

Ongoing studies are attempting to relate hormonal receptors in the various stages of tumor growth, as well as to define immunologic parameters influencing tumor take, growth, and control. Further biochemical characterization especially concerning polyamines in this tumor system is also being done. Finally, the timing, dose schedule, and types of non-hormonal chemotherapy drugs at all phases of hormonal and non-hormonal growth of this tumor are being studied.

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Some New Approaches to Potential Test Systems for Drugs Against Prostatic Cancer [1]

A. A. Sandberg,* J. Müntzing, N. Kadohama, J. P. Karr, G. Sufrin, R. Y. Kirdani, and G. P. Murphy [2]

SUMMARY

We have previously reported on test systems, based on 5α-reductase (5α-RA) and arginase activities and steroid deposition in animal prostates, potentially useful in screening drugs possibly effective in cancer of the prostate. Recently, we have concentrated on the development of other in vivo and in vitro systems which may prove of further value in testing such drugs. These systems include the following: (a) The effects of drugs on 5α-RA activity in human and animal non-malignant and human cancerous prostatic tissues in organ culture. The parameters necessary for the maintenance of optimal 5α-RA in such explants have been determined, and it has been shown that certain agents (estramustine phosphate, progesterone, estradiol-17β) can inhibit 5α-RA under in vitro conditions, pointing to the potential use of such an approach in screening various cytostatic agents. In addition, ^{65}Zn deposition, the histology, and the androgen metabolism in such tissues in organ culture are being determined as additional parameters. (b) The deposition of ^{65}Zn in the rat dorsolateral gland, particularly as affected by prolactin and testosterone, and the effects of chemotherapeutic agents on such deposition. Methotrexate and CCNU have been shown to be potent inhibitors of ^{65}Zn deposition in the dorsolateral gland. The parameters related to zinc metabolism in the prostate are being further investigated. (c) The demonstration of receptors for estrogens (estradiol-17β, diethylstilbestrol) in the prostate of the baboon. The various parameters related to the specificity of such receptors have been established. The development and standardization of these approaches, some facets of which are reported in the present publication, may prove them to be more sensitive, specific, and optimal than other systems and should afford an opportunity to test various combinations of drugs potentially useful in cancer of the prostate with relative speed, efficacy, and ease of manipulation.

[Cancer Treat Rep 61:289–295, 1977]

The development of a variety of in vivo and in vitro test systems for drugs potentially useful in cancer of the prostate is necessitated not only by the lack of a readily available animal model system, but also by the variations of the human disease (1). In the past, we have reported (1–3) on several such test systems which depended on certain unique properties of the prostatic gland (ie, the response of prostatic 5α-reductase [5α-RA] and arginase activities in the rat and the effects on the deposition of labeled estrogens and androgens in the dog prostate following the administration of various drugs to the animals) and on the rationale for using these systems in testing drugs possibly of value in human prostatic cancer. Results with these test systems, along with some of those of others, have been used by the National Prostatic Cancer Project in deciding upon chemotherapeutic regimens to be used in prostatic cancer. Nevertheless, several shortcomings of some of these in vivo systems (length of time required for prostatic effects, amounts of drugs needed, availability of appropriate animals, etc) and a natural search for systems which may prove to be

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TABLE 1.—5α-RA in ventral and dorsolateral rat prostate in short-term organ culture*

System	Picomols of T converted/30 mg of tissue (average of duplicates)				
	T	A-dione†	A-diol†	DHT	A-diol + DHT
Ventral					
No hormone	0.051	0.002	0.020	0.085	0.105
5×10^{-7} M T	52.08	1.03	2.91	6.03	8.94
Dorsolateral					
No hormone	0.086	0.003	0.011	0.080	0.091
5×10^{-7} M T	44.16	0.20	0.72	0.73	1.45

*Explants were prepared from ventral and dorsolateral lobes of normal adult rats. After 1 day in hormone-less medium, 5×10^{-7} M T was added to 1 set of culture dishes. There were 2 such sets each for the ventral and dorsolateral tissues. After maintenance in hormone-less or T-containing medium for 2 days, the explants were exposed to 0.1 microcurie/ml of ^{3}H-T for 2 hrs for assay of 5α-RA.

†A-dione = androstenedione; A-diol = androstanediol.

more optimal or specific than existing ones, prompted us to undertake the development of some new approaches. This presentation deals with a preliminary description of these systems, still utilizing properties peculiar to the prostate as end-points in the testing of drugs.

"ORGAN CULTURE" OF PROSTATIC TISSUE

In this approach, we initially investigated the conditions necessary for the maintenance of 5α-RA activity in the prostatic tissue under "organ culture" conditions. Since this enzyme plays a crucial role in prostatic physiology, ie, the conversion of testosterone (T) to dihydrotestosterone (DHT),[3] it was felt that the maintenance of 5α-RA would be reflective of a "healthy" physiologic status of the tissue. It is recognized that the testing of various chemotherapeutic agents in vitro in this "organ culture" system may not be applicable to some of these agents; however, success with some of these agents would prove its usefulness as a test system and, ultimately, its applicability in testing chemotherapeutic agents against human cancerous tissue under similar conditions.

Materials and Methods

For most experiments, adult male Wistar rats, about 400–450 g in weight, were used as a source of prostatic tissue. In one experiment, prostates were obtained from 28-day-old Fischer rats. For growth in organ culture, the ventral and/or dorsolateral lobes of the prostate were utilized and the 5α-RA in these tissues was determined by previously described methods (1–3).[3]

Results

In performing the 5α-RA assays, as applied to the effects of drugs and other compounds, we chose to rely on the stimulating effect of T only, since the effects of added insulin and/or cortisol were not consistent. Trials with various concentrations of T led us to use 5×10^{-7} M as the optimal dose with the ventral prostate of the rats. The results are shown in table 1 and figure 1. The stimulatory effect of T and some inhibition of this effect by 5 μg/ml of estradiol-17β (E_2) or progesterone (P) are evident in both the ventral and dorsolateral prostates.

Figure 2 shows results obtained with two human cancers of the prostate. Stimulation of 5α-RA brought about by the presence of T is evident. A significantly higher activity was observed with 5×10^{-6} M T than with the lower concentration in explant No. 2. In addition, inhibition by estramustine phosphate occurred with 10 μg/ml of the compound in the organ culture of specimen No. 1.

^{65}ZINC DEPOSITION IN THE PROSTATE

In the present study on rats, our aim was to further examine the effects of estrogens and other hormonal factors on the prostatic zinc uptake and the manner by which zinc is bound in the prostate (4), with the hope of utilizing this system as a

[3]Kadohama N, Kirdani RY, and Sandberg AA. 5α-Reductase as a target enzyme for anti-prostatic drugs in organ culture. In preparation.

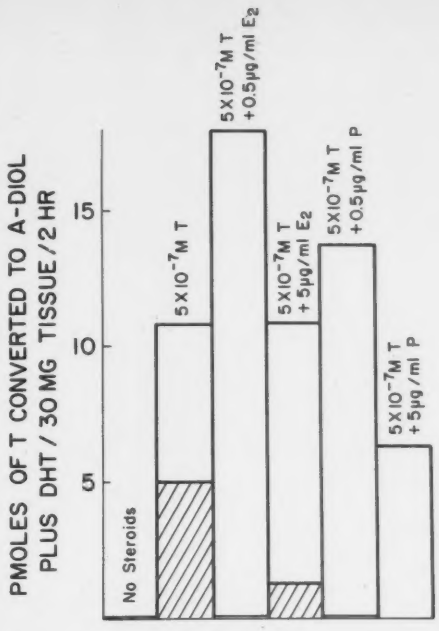

FIGURE 1.—Effects of T, E_2, and P on the 5α-RA of the rat prostate in organ culture. Prostatic explants were derived from normal adult rats. After 1 day in hormone-less medium, the tissues were exposed to various steroids for 6 days, with the media being changed every 2 days. On the 7th day, 0.1 microcurie/ml of ^{3}H-T was added for 2 hrs for the assay of 5α-RA. The higher 5α-RA in tissues from the ventral prostate as compared to that from the dorsolateral gland is evident. Inhibition of 5α-RA by the higher concentrations of E_2 and P is shown.

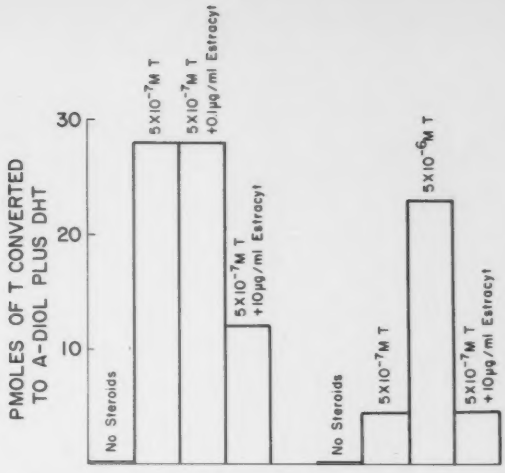

FIGURE 2.—5α-RA in explants prepared from 2 human cancers of the prostate. The explants were maintained for 4 days in hormone-less medium or in the presence of T with and without estramustine phosphate. The medium was changed every 2 days and on the 5th day the explants were pulsed with ^{3}H-T (0.1 microcurie/ml) for 2 hrs. Inhibition of 5α-RA in explant No. 1 (right part of the figure) by 10 µg/ml but not by 0.1 µg/ml of estramustine phosphate is shown. Explant No. 2 (left part of the figure) was stimulated to higher 5α-RA by 10^{-6} M compared to 10^{-7} M T. Inhibition of 5α-RA by 10 µg/ml of estramustine phosphate is evident.

sensitive and reliable index for the effects of chemotherapeutic agents.

Materials and Methods

Details of the various procedures used and the injection schedules have been described elsewhere (4).

Results

The radioactivity concentrations in tissues from rats killed at different times after injection of $^{65}Zn^{++}$ are presented in figure 3. In contrast to the other tissues, the dorsolateral prostate showed a gradual concentration increase over the whole time period; the increase was relatively rapid between 8 and 24 hours after injection, but later the concentration increase was not that prominent. Since the greater part of the zinc was bound to the dorsolateral rather than the ventral prostate within 24 hours after injection, the uptake in the dorsolateral gland 24 hours after injection of the ^{65}Zn was used in the experiments described below.

The zinc uptake in the dorsolateral prostate was considerably reduced by orchiectomy (fig 4). The concentration of radioactivity was significantly higher in orchiectomized rats treated with estradiol benzoate than in the orchiectomized control rats ($P < 0.05$). The prolactin release inhibitor (Compound A) seemed to counteract the effects of estradiol benzoate on the zinc uptake in the dorsolateral prostate of orchiectomized rats. Adrenalectomy in orchiectomized rats had no effect on the concentration of radioactivity beyond the castration induced decrease. Estradiol benzoate did not increase the zinc concentration in the dorsolateral prostate of adrenalectomized orchiectomized rats. The radioactivity concentration in the ventral prostate was not significantly changed by orchiectomy alone or combined with adrenalectomy; neither estradiol benzoate nor the prolactin release inhibitor induced any significant changes.

One of the three prolactin release inhibitors (Com-

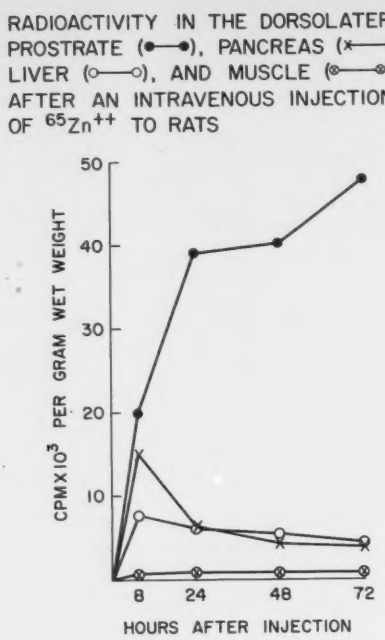

FIGURE 3.—^{65}Zn concentrations with time in various tissues of the rat. The dorsolateral prostate has the highest amount of radioactivity, which appeared to peak at about 24 hrs after the iv injection of the ^{65}Zn (5 microcuries).

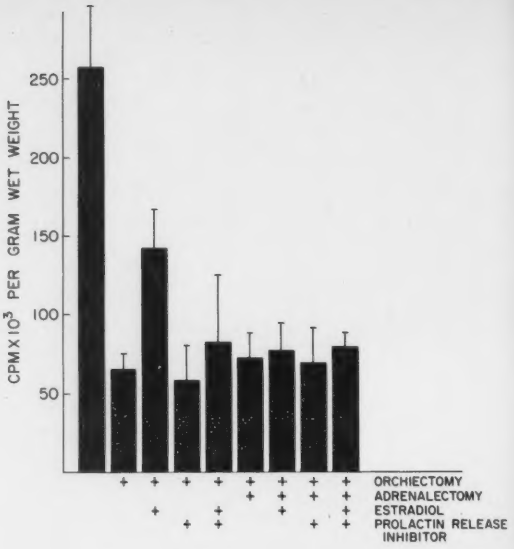

FIGURE 4.—Effects of orchiectomy, adrenalectomy, E_2 (1 mg/day), and a prolactin release inhibitor (Compound A of table 2; 0.1 mg/day) on the concentrations of ^{65}Zn in the dorsolateral prostate of castrated rats. E_2 and the inhibitor were injected iv daily for 7 days and 25 microcuries of ^{65}Zn given on Day 6.

pound B) caused a significant decrease of the ^{65}Zn concentration in the dorsolateral prostate ($P < 0.05$) (table 2). There was no change in the weight of the gland. The two other prolactin release inhibitors had no effect on radioactivity concentrations or gland weight. Cyproterone acetate significantly reduced the weight of the dorsolateral prostate ($P < 0.05$) but did not affect the zinc concentration. The radioactivity concentration in the ventral prostate was not changed by any of the treatments. Cyproterone acetate caused a significant weight decrease of the gland.

The schedule of administration and the effects of chemotherapeutic agents on body and prostatic weights and on the uptake of labeled zinc by the ventral and dorsolateral prostate of the rat are shown in table 3 and figure 5. Significant weight decreases were induced by 5-fluorouracil (5-FU) and methotrexate and an increase was induced by cyclophosphamide in the dorsolateral gland; significant decreases were caused by 5-FU and estramustine phosphate and increases by cyclophosphamide and Leo-1031 in the ventral prostate. Significant changes in ^{65}Zn uptake were produced in the dorso-

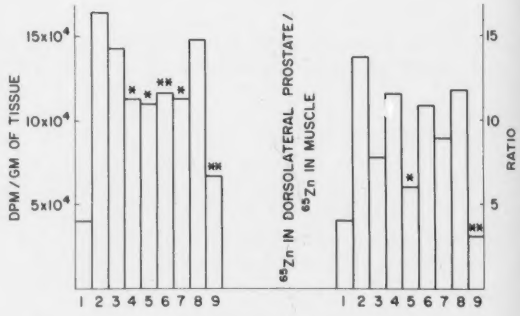

FIGURE 5.—Effects of various drugs on the concentration of ^{65}Zn in the dorsolateral gland of castrated rats maintained on an androgen. For details of the experiment see table 3. A double asterisk (**) indicates $P > 0.01$ and a single asterisk (*) indicates $0.05 > P > 0.01$. Methotrexate (No. 9) produced the most prominent inhibition of ^{65}Zn uptake, followed in this effect by CCNU, estramustine phosphate, cyclophosphamide, and Leo-1031.

TABLE 2.—Effects of prolactin release inhibitors and cyproterone acetate on ^{65}Zn uptake by prostates of intact animals*

Treatment	No. of animals	Body weight (g)		Wet weight (mg) of—		^{65}Zn concentration (relative to the concentration in muscle) in—	
		Initial	Final	Ventral prostate	Dorsolateral prostate	Ventral prostate	Dorsolateral prostate
—	5	372 ± 7	393 ± 12	495 ± 27	309 ± 21	1.67 ± 0.33	19.85 ± 0.68
Compound A (0.1 mg/day)	5	373 ± 20	374 ± 22	493 ± 39	320 ± 14	1.33 ± 0.09	21.53 ± 1.59
Compound B (0.1 mg/day)	5	368 ± 26	397 ± 9	556 ± 34	324 ± 13	1.32 ± 0.12	12.77 ± 0.56
Compound C (0.05 mg/day)	5	397 ± 17	405 ± 16	520 ± 60	315 ± 25	2.85 ± 0.91	20.19 ± 2.04
Cyproterone acetate (3 mg/day)	5	379 ± 11	364 ± 11	341 ± 41	235 ± 11	1.94 ± 0.19	15.75 ± 1.88

*Effects of 3 prolactin release inhibitors, 6-methyl-8-pyridiniummethyl-ergolene tosylate (Compound A), 6-methyl-8-ergolenylacetamide (Compound B), and 8-acetanilinomethyl-6-methyl-8-ergolene (Compound C), and cyproterone acetate on prostatic wet weight and concentration of ^{65}Zn. Cyproterone acetate was injected for 8 days and the prolactin inhibitors for 17 days. $^{65}Zn^{++}$ (25 microcuries/animal) was injected 24 hrs before the animals were killed on the day after the last injection. The mean values and their standard errors are given.

TABLE 3.—Effects of drugs on prostatic parameters*

Treatment	Daily dose (mg/kg)	Body weight (g)—		Body weight change (g)	Ventral prostate weight (mg)	Dorsolateral prostate weight (mg)
		At the start of the injections	At termination			
No androgen (oil only)	—	383 ± 9	333 ± 7	−50 ± 2	74.3 ± 0.05	62.3 ± 0.01
Saline + A	—	434 ± 18	432 ± 18	1 ± 2	365.2 ± 32.3	295.7 ± 15.3
5-FU + A	30	399 ± 15	345 ± 13	−55 ± 9**	272.1 ± 31.8	206.9 ± 4.5**
Cyclophosphamide + A	18	422 ± 20	379 ± 15	−43 ± 7**	505.1 ± 36.0*	354.7 ± 18.7*
Estramustine phosphate + A	100	429 ± 10	374 ± 8	−55 ± 2**	258.6 ± 15.1*	255.7 ± 16.0
CCNU + A	5	419 ± 14	425 ± 13	6 ± 2	373.1 ± 17.6	316.2 ± 25.0
Leo-1031 + A	50	450 ± 10	375 ± 5	−75 ± 8**	465.4 ± 12.5*	313.9 ± 22.5
Streptozotocin + A	15	401 ± 22	380 ± 23	−22 ± 6**	390.8 ± 39.2	271.8 ± 15.9
Methotrexate + A	0.75	392 ± 13	354 ± 13	−38 ± 12**	355.9 ± 20.2	242.0 ± 4.0**

Body weight and weights of ventral and dorsolateral prostates in orchiectomized rats injected im with a long-acting androgen (A) (testosterone hexyloxyphenylpropionate, Andradorin) on Day 14 after operation, and ip with various cytostatic agents once daily on Days 14–21. ^{65}Zn (25 microcuries/animal) was injected at the same time as the last ip injection and the animals were killed 24 hrs later. There were 5 animals per group. The mean values and their standard errors are given. A significant difference between the body weight change or the weight of the prostates in the control animals and those in the experimental animals is shown by "" ($0.05 > P > 0.01$) or "**" ($P < 0.01$).

lateral gland by the administration of methotrexate and CCNU and to a lesser extent by cyclophosphamide, estramustine phosphate, and Leo-1031. When expressed in terms of the radioactivity present in muscle, a significant change was seen in the ventral and dorsolateral prostates following the administration of methotrexate and estramustine phosphate, resulting in a significantly lower concentration of the labeled zinc than in the controls.

ESTROGEN BINDING BY THE BABOON PROSTATE

The use of estrogens in the treatment of cancer of the prostate grew out of the demonstration of their regressive effects on the gland (5), assumed to be mediated indirectly through suppression of the hypothalamic-pituitary-testis axis (6). Direct effects of estrogens on the prostate, however, have been reported and these include a number of different metabolic and biochemical parameters (3).[4] The effects of estrogens on the uptake of labeled zinc by the dorsolateral gland of the rat prostate have been reported in a previous section of this paper. The presence of receptor proteins for estrogens in the rat prostates and in the human non-cancerous and cancerous gland has been reported (7,8). Since cytoplasmic receptors appear to be essential to the action of steroids in some target tissues, we investi-

[4]Karr J, Sufrin G, Kirdani RY, et al. Prostatic binding of estradiol-17β in the baboon. In preparation.

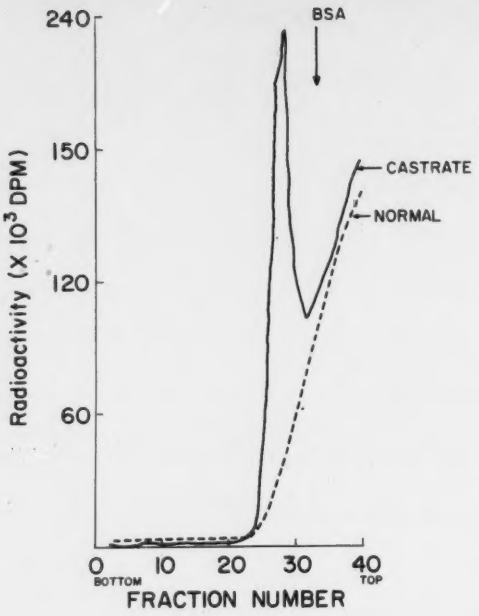

FIGURE 6.—SDGC of cytosols from the caudal lobe of a normal (intact) and a castrated baboon, respectively, incubated with ^{3}H-E_2 in vitro. A definite receptor peak is evident with the caudal lobe cytosol from the castrated baboon, whereas such a peak could not be demonstrated with the cytosol of a normal animal. The latter may be due to the inability of the added ^{3}H-E_2 to displace endogenous steroids from receptor sites, which would be vacant in the cytosol of a castrated animal.

gated the existence and properties of estrogen receptors in a primate model, the baboon; the results may have direct application to the human gland, including cancer of the prostate.

Materials and Methods

The injection techniques, preparation of tissues, determination of radioactivity, and various binding parameters were performed according to methods previously published from this and other laboratories (1–3).[4]

Results

After in vivo infusion with ^{3}H-E_2 for 1 hour, cytosol from the prostate was prepared and the unlabeled compounds to be tested (DHT, T, E_2, and diethylstilbestrol [DES]) were added in a 100-fold excess. Neither T nor DHT significantly displaced bound labeled E_2, whereas radioinert E_2 and DES successfully competed with the labeled E_2 for receptor sites.

When cytosol was obtained from the prostate of intact animals it was difficult to demonstrate the existence of peak radioactivity due to the presence of endogenous steroids. However, when similar preparations were processed from animals which had been castrated 40 hours prior to prostatectomy, definite peaks for ^{3}H-E_2 following sucrose density gradient centrifugation (SDGC) were observed (fig 6). The addition of radioinert E_2 in 100-fold excess or 3.0 μg of nafoxidine invariably abolished the peak of radioactivity in the cytosol. DHT and T affected this peak only slightly.

Since castration facilitated the in vitro identification of cytosol binding of ^{3}H-E_2, the effect of castration followed by exogenous replacement with T or T plus E_2 on prostatic binding was studied. Animals were castrated and 48 hours later started on T-propionate injections (2 mg/kg/day, sc), or the same course of T combined with E_2-valerate (100 μg/kg/day, sc). The injections were given daily for 3 days and the prostates removed 24 hours following the final injection. Cytosol was prepared in the usual manner, incubated with ^{3}H-E_2 for 90 minutes at 4°C, and SDGC performed. A peak of radioactivity with a value of 4.2 was obtained, as in previous experiments. Only a slight reduction in peak radioactivity in the prostates of animals pretreated with T plus E_2 was observed.

DES and estramustine phosphate were shown to successfully compete for the binding site in the cytosol of the caudal prostate of the baboon; when ^{3}H-estramustine phosphate (0.015 microcurie) or ^{14}C-DES was incubated with such cytosol, definite peaks of radioactivity following SDGC with a value of 4.2S were observed. Translocation of the cytosol E_2 receptor to the nucleus was demonstrated.

DISCUSSION

Organ Culture of Prostatic Tissue

One of the salient features of the work done so far has been the demonstration of a definite effect of T on 5α-RA in human cancerous tissue from the prostate maintained in organ culture. This restoration of 5α-RA by T (to about 30% of the level found in normal or tumor tissues prior to organ culture) can be affected by certain antitumor agents and points to the potential of this system for screening drugs possibly useful in prostatic cancer. This is now being undertaken in our laboratory. Even though the study has thus far been concentrated on 5α-RA, the effects on ^{65}Zn deposition in vitro, the histologic

picture of the tissues, and the androgen metabolism by the prostatic explants in organ culture have also been determined and will be reported in the near future.

^{65}Zn Deposition in the Dorsolateral Prostate of the Rat

The exact role of zinc in the prostate remains to be elucidated, though undoubtedly it plays a crucial role in the function of the gland. Hormonal control of zinc in the prostate has been demonstrated in the present studies, which point to T, E_2, and prolactin as playing a role in such control. It is possible that E_2 produces its effect through prolactin, and the control by the latter of the ingress of zinc into the prostatic cells may be related, in part, to the increased concentration of intracellular T induced by prolactin. The effects of some of the chemotherapeutic agents on ^{65}Zn deposition in the studies presented point to the potential of this system, particularly since it is based on a parameter somewhat different from that of other approaches used to date.

Estrogen Binding in Baboon Prostate

The specificity of the cytosol receptor in the prostate of the baboon is indicated by the inability of T and DHT to displace the labeled E_2 from the receptor, either in vitro or in vivo. On the other hand, the labeled E_2 was readily displaced by carrier E_2, DES, and nafoxidine, indicative of the affinity of the receptor for those substances which are thought to compete with labeled E_2 at specific receptor sites. In addition, if such binding were not specific or were due to contamination of testosterone binding globulin (TeBG) or other plasma proteins with high affinity for E_2, one would expect displacement by T and DHT. Clearly, however, this was not the case. It has also been shown that DES does not displace E_2 bound to TeBG (9,10). This is not the case with E_2 bound to cellular receptors, eg, in breast tissue or to the receptor in our studies, in which DES readily displaced E_2. Another important indication of the specificity of the E_2 receptor was the observation based on the in vivo studies following castration, in which large doses of T were administered to the castrated animals without significantly affecting the receptor peak in the cytosol of the prostate.

The demonstration in the baboon prostate of a receptor for E_2 which shows considerable specificity towards this steroid may be related to the localization of labeled estrogens within the prostate of this animal. The exact function of this receptor protein and the estrogen associated with it has yet to be elucidated. Furthermore, it is inconceivable that they do not play a crucial role in the metabolism and integrity of the prostatic cells, E_2 receptors also having been described in the rat prostate and in human non-cancerous and cancerous glands (7,8), and agents which would affect this protein and/or binding of estrogens to it should be considered of some value in therapy of cancer of the prostate. It is our intention to test various agents for their effects on the receptor in the prostate and on the association of labeled estrogens with such a protein. However, the demonstration in the present study of translocation of the cytosol E_2-complex to the nucleus may point to transcriptional effects of E_2 in the prostate.

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Evaluation of Extended-Field Radiotherapy for Prostatic Neoplasm: 1976 Progress Report [1]

Malcolm A. Bagshaw,[2,*] David A. Pistenma,[2] Gordon R. Ray,[3] Fuad S. Freiha,[2] and Richard L. Kempson [2]

SUMMARY

These observations show that short-term, disease-free survival is (*a*) excellent (86.5%) in patients with negative lymph node biopsies, (*b*) greatly diminished (30%) in patients with positive para-aortic and pelvic lymph node biopsies, and (*c*) intermediate (71.5%) in patients with positive pelvic lymph node biopsies only in the pelvic region following extended-field radiation therapy of carcinoma of the prostate.

[Cancer Treat Rep 61:297–306, 1977]

During the years from 1956 to 1971, in a group of 230 patients with carcinoma of the prostate localized within the capsule and treated by external beam irradiation to the prostate and to the periprostatic region only, we reported that the actuarial survival was 72% at 5 years and 44% at 10 years. In a concomitant series of 200 patients who were similarly treated but who had extracapsular extension of the tumor, the actuarial survival was 51% at 5 years and 38% at 10 years (1). In neither group was there roentgen evidence of distant metastases at the time of radiation therapy; the distinction between those with disease limited to the prostate and those with extracapsular extension was based solely upon digital examination of the prostate (1–4). During the evolution of this study, it was proposed that an explanation for the significant difference in survival might have been the failure to recognize and treat either frank regional adenopathy or potentially involved lymph nodes. Thus, it was speculated that a treatable sub-group of patients may exist intermediate between those with the neoplasm still localized at the primary site and those with either osseous or other more distant metastases. It seemed likely that this group of patients might have dissemination to regional lymph nodes only. If this were true, especially for the group with extracapsular extension, all of the neoplasm would not have been included in the treatment volume. By design, the treatment volume in this earlier series was confined to the prostate and the periprostatic region because in the early megavoltage era, tolerable tissue doses and treatment volumes were not well established.

Beginning in 1971, a prospective clinical trial aimed at a more thorough evaluation of the extent of disease before treatment and the determination of the efficacy of treatment of involved and potentially involved lymph nodes began. In the previous report, 34 patients had been studied; this report enlarges the group to 66 patients.

PROTOCOL SUMMARY

Requirements

Patients may qualify for inclusion in the protocol if they have biopsy-proven carcinoma of the prostate and are ≤ 70 years of age, have a negative Technetium-labeled-pyrophosphate bone scan, and have a negative skeletal roentgen survey for bone metastases. Candidates who pass the initial screening then have an intravenous pyelogram and a lower extremity lymphogram. This is followed by a retroperitoneal pelvic exploration for the direct

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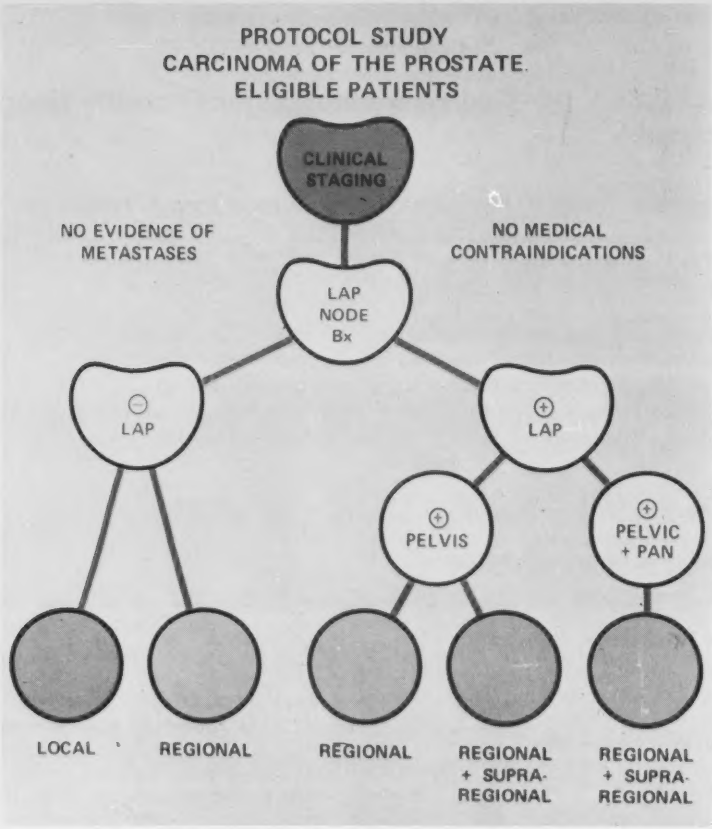

FIGURE 1.—Extent of lymph node metastases is determined by laparotomy (LAP) and retroperitoneal node dissection. If no metastases are present (⊖LAP), patients are randomly assigned to either local prostatic treatment only, or treatment to the prostate region + pelvic lymph node region. If the LAP is positive for adenopathy (⊕LAP), but only in the pelvic region (⊕pelvis), patients are randomly assigned to either treatment to the prostate and pelvic lymph node region only (regional), or to the entire pelvic region including the prostate + the next higher echelon of lymph nodes, the para-aortic nodes (regional + supra-regional). If the para-aortic nodes are positive as well (⊕pelvic + para-aortic nodes), then all known areas of involvement are treated without randomization (regional + supra-regional).

evaluation of lymph node involvement. Additional studies have included Jamshidi and direct bone biopsies and serum and bone marrow acid phosphatase determinations.

Therapeutic Options

A scheme of the therapeutic options is presented in figure 1. The radiation treatment used is external beam megavoltage irradiation generated by a 4-MeV linear accelerator. In the treatment of the prostate only, the radiation is delivered by means of left and right 120° moving beam arc therapy to a central tumor dose of 7000 rads in 7 weeks (figs 2 and 3). When both the pelvic lymph nodes and the prostate are treated, an extended four-field technique (anterior, posterior, right and left lateral) is used which encompasses the prostate and its regional lymph node stations in continuity (figs 4 and 5). For this extended pelvic treatment, 2600 rads at the rate of 1000 rads/week are delivered to the large volume (figs 4 and 5); then the treatment volume is contracted to the prostatic region only, for an additional 2000 rads delivered over a period of 2 weeks. For the reduced volume, the treatment is 120° arc therapy confined to the prostate only, as described above. The treatment volume is then re-expanded and an additional 2400 rads are delivered to the extended field which, of course, includes all of the pelvic lymph nodes and the prostate as well. This

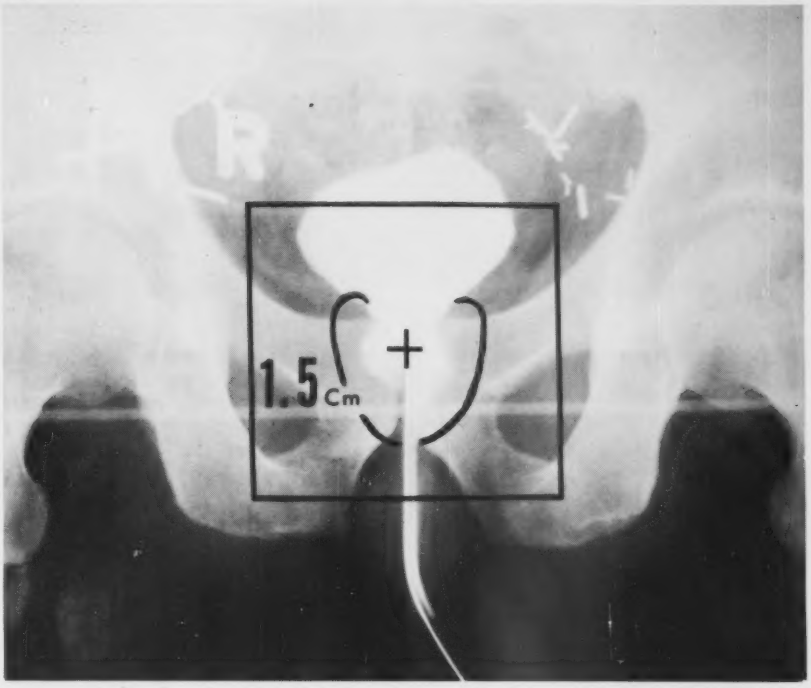

FIGURE 2.—Alignment position for the small prostate field in the frontal projection is indicated by the black square. The field size measures 8 × 8 cm at the isocenter. Note contrast in the bladder and in the prostatic urethra.

technique extends the overall time for the treatment of the pelvic lymph nodes to 7 weeks, permitting a 2-week period for recovery of any loops of small bowel which might be included within the treatment volume. In the meantime, the prostate receives continuous therapy to a total of 7000 rads in 7 weeks. Treatments are given at the rate of 200 rads of tumor dose/day, and it is seldom necessary to vary from this formula. Technical points of importance include trimming the anterior and posterior fields in such a way as to be certain that all relevant lymph node sites are included but that exposure of bone marrow is minimized. Notice in the lateral view (fig 5) that the posterior margin is adjusted to include only about one half of the rectum. It is thought that preservation of the posterior wall of the rectum, at least during the treatment of the lateral fields, is important for minimizing rectal radiation reactions. It is also important that all treatment fields are treated each day, at least for photon energies from about 2 to 8 MeV. This assures a consistent, homogenous absorption of radiation throughout the treatment volume and greatly improves patient tolerance. Rectal and small bowel symptoms are minimized, and suprapubic, penile, or scrotal edema, which can be seen with simple parallel-opposed anterior posterior fields, is never seen. The figures illustrate only the lower end of the para-aortic field, but this field extends to the top of T12 and includes all of the para-aortic lymph nodes. Its position and size are determined with the aid of contrast material in the kidneys in order to avoid excessive exposure to the kidneys. The para-aortic regions are treated with anterior posterior and posterior anterior cross-fire to 4000 rads only, and an additional 1000 rads are added either by left and right shallow anterior obliques which traverse anterior to the kidneys or by sector rotation. The reason for maintaining the anterior posterior cross-fire in the para-aortic region is to gain whatever prophylactic advantage against bone metastases might be obtained by including the lumbar spine to at least the first 4000 rads. The split-course technique used in the pelvis is also used in the para-aortic region. It

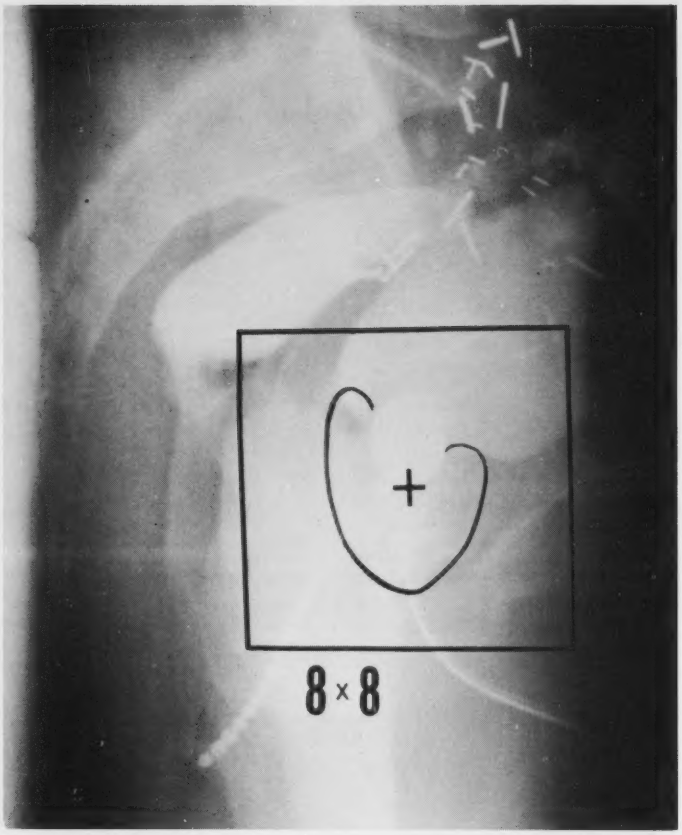

FIGURE 3.—Alignment position for the small prostate field as projected in the lateral view. Note the mercury-filled Foley catheter which denotes the position of the prostatic urethra. The bladder and rectum are also filled with contrast agents. The radio-opaque beads demonstrate the position of the anus.

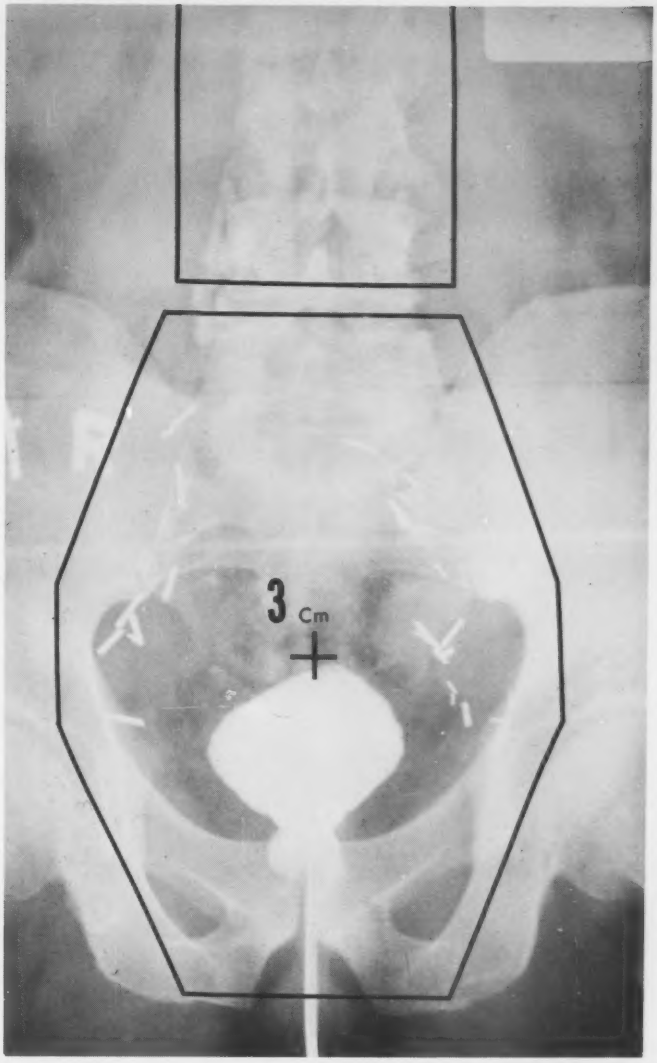

FIGURE 4.—Alignment and field cross section for the extended pelvic and para-aortic fields. 3 cm indicates the shift required in this instance for aligning the central axis of the preliminary localization beam with the desired central axis of the final treatment field. The position of the lymph nodes is indicated by the silver clips. Contrast material is in the bladder and prostatic urethra.

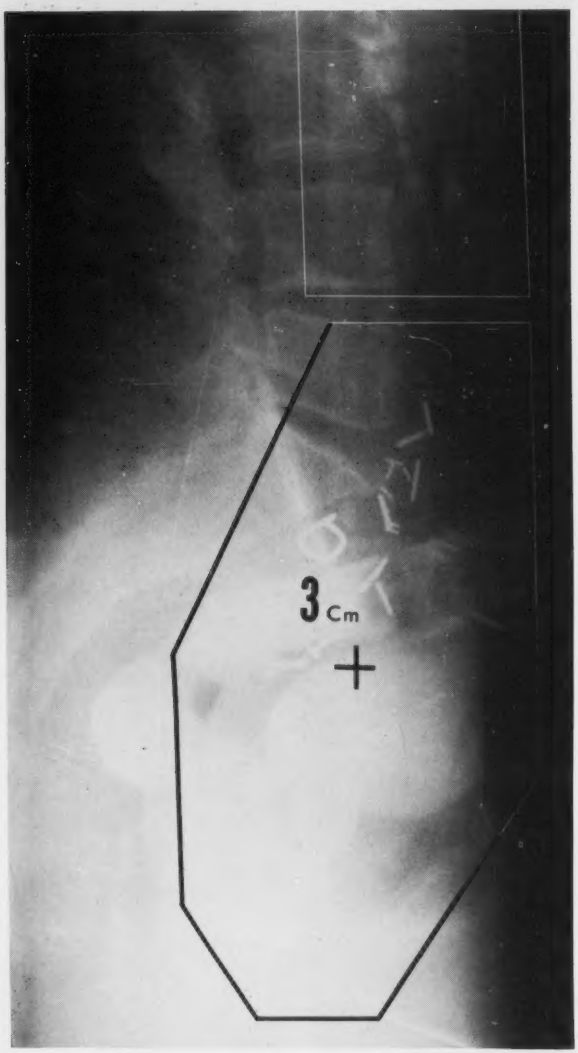

FIGURE 5.—Lateral alignment and beam shaping required for the right and left lateral extended-field treatments. Radio-opaque contrast demonstrates the position of the urinary bladder, the rectum, and the prostatic urethra. The position of the pelvic lymph nodes is indicated by silver clips. The para-aortic lymph nodes are visible overlying and anterior to the vertebral bodies because of retained Ethiodol contrast from a prior lymphogram.

has been possible in most patients to treat the para-aortic region concomitantly with the extended pelvic field.

Modification of the Initial Protocol

In the course of this study several refinements or modifications in the initial research plan have been necessary.

(*a*) A retroperitoneal node dissection has replaced the original transperitoneal approach because an unacceptably high incidence of radiation enteritis occurred initially. For example, 12 instances of this complication were noted in the first 24 patients entered in the protocol, eight of whom required subsequent exploration and resection of stenotic areas. No small bowel obstructions have occurred in the most recent 21 patients, all of whom had retroperitoneal dissections. Although the dramatic reduction in radiation enteritis has been extremely gratifying, a disadvantage of the retroperitoneal dissection is the inability to sample high para-aortic nodes. Initial data on the high incidence of para-aortic adenopathy (tables 1 and 2) were revealing, and it is expected that this incidence will diminish with the more restricted surgical procedure. Nevertheless, even with the retroperitoneal approach, the most inferior para-aortic nodes can still be sampled.

(*b*) Several modifications of radiotherapy technique were also directed toward a reduction in small bowel complications. These include: a reduction in total dose to the lymph node areas from 5500 to 5000 rads; a 2-week rest period at the mid-point of irradiation of the extended fields; and the adoption of a firm treatment policy which required the treatment of all fields every day.

(*c*) An essentially complete node dissection has been substituted for sampling of suspicious nodes. This modification was adopted after discovering that the more complete retroperitoneal dissection followed by aggressive radiation treatment apparently does not increase overall morbidity and provided considerably greater information on the extent of lymph node involvement. More recently, some transitory supra-pubic and genital edema has occurred in patients who received the more aggressive lymph node dissections.

(*d*) The bone biopsies have been discontinued because 67 percutaneous and 41 open biopsies failed to demonstrate a single metastasis to the bone.

Thus, the cross correlation of the lymphograms, the surgical staging of the lymph nodes, the serum acid phosphatase, the extent of the primary neoplasm, and the degree of pathologic differentiation of the primary neoplasm remain significant parts of the overall study.

Subsequent long-term evaluation will include assessment of the status of the primary neoplasm by periodic digital examinations, repeat needle biopsy of the primary site at about 15 months after radiotherapy, serial bone scans, serial acid phosphatase determinations, and other observations consistent with a comprehensive long-term followup program.

TABLE 1.—Overall comparison of lymphogram and lymph node biopsy results*

Comparison	Total No. of patients (%)	
Agreement		
Both studies normal	33 (55.0)	
Both studies abnormal	11 (18.3)	
Pelvic nodes only		7 (11.7)
Pelvic and para-aortic nodes		4 (6.7)
	44 (73.3)	
Discrepancies		
Lymphogram (+), lymph node biopsy (−); (false +)	3 (5.0)	
Lymphogram (−), lymph node biopsy (+); (false −)	8 (13.3)	
Lymphogram (+) in pelvic nodes, lymph node biopsy (+) in pelvic and para-aortic nodes	5 (8.3)	
	16 (26.7)	
Total	60* (100)	

*4 of the 66 patients who had lymph node biopsies had technically inadequate lymphograms, and 2 other patients had inadequate sampling of lymph nodes for comparison with their lymphograms.

TABLE 2.—Incidence of lymph node involvement by prostatic cancer (66 patients)

Lymph node region	Total No. of patients biopsied	No. of patients with cancer (%)
Para-aortic	54	12 (22.2)
Common iliac	52	11 (21.2)
External iliac	48	13 (27.1)
Internal iliac	44	14 (31.8)
External or internal iliac or both	56	20 (35.7)
Obturator	28	11 (39.3)

RESULTS
(Update of results since the beginning of the protocol in 1971)

Diagnostic Variables

Comparison Between Lymphogram and Lymph Node Biopsy Results

To date, 66 patients have been studied and of these, 60 are available for comparison of lympho-

gram and lymph node biopsy results and 61 are available for post-treatment evaluation. Five patients are not included in the evaluation of current status either because they did not complete therapy or because they are still undergoing therapy.

A comparison of the lymphogram interpretations with lymph node biopsy results (table 1) shows a 73.3% correlation between the two types of diagnostic studies. The 26.7% discrepancy is due to (*a*) three patients with abnormal lymphograms not confirmed by biopsy; (*b*) eight patients with negative lymphograms who had positive pelvic node biopsies (five of these eight had foci of tumor in lymph nodes too small for roentgenographic detection); and (*c*) five patients whose lymphograms were positive in the pelvic nodes but who also had biopsy-positive para-aortic nodes which were not detected by lymphography. Since the lymphograms were partially positive in these patients, they could be added to the 44 patients in whom there was total agreement. If this was done, there would be 49 patients (81.7%) with agreement opposed to only 11 patients (18.3%) with discrepancies. These findings continue to support the position that lymphography is valuable in terms of well-defined, positive lymph nodes, but inconclusive where the degree of involvement is either microscopic or insufficient to cause characteristic changes in the lymph nodes.

An analysis of biopsy results by anatomic distribution is presented in table 2. This represents a conservative estimate of the number of nodes involved and the sites of involvement because many of the early patients in this series had less than complete node dissections. The relatively high incidence of para-aortic, common iliac, and external iliac metastases was surprising.

Clinical Staging Versus Surgical Staging

Table 3 presents a comparison of the clinical staging with the results of lymph node biopsy. Clinical examination demonstrated disease limited to the prostate (DLP) in 33 patients and extracapsular extension (ECE) in 33. In the group of 33 DLP patients, a biopsy showed positive pelvic lymph nodes in five patients and positive pelvic and para-aortic lymph nodes in two. In the group of 33 ECE patients, a biopsy showed positive pelvic lymph nodes in ten patients and positive pelvic and para-aortic lymph nodes in ten. Of the ten patients with positive pelvic lymph node-only biopsies, four had abnormal lymphograms. However, of the ten patients with positive para-aortic lymph node biopsies, eight had abnormal lymphograms. Five of the eight lymphograms were positive only in the pelvis and three were positive in both pelvic and para-aortic lymph nodes. One lymphogram was normal and one was technically inadequate.

Histopathology

The distribution of patients according to histologic grade of the primary tumor and clinical stage is shown in table 4. The histopathology in each patient is reviewed by members of the Surgical Pathology Division at Stanford prior to the initiation of radiation therapy, and all specimens are subsequently systematically reviewed by Dr. Kempson. The neoplasms are graded from the most well differentiated through the least differentiated in four grades (I–

TABLE 3.—Correlation of clinical stage with lymph node biopsy results

Clinical stage		Results of lymph node biopsy				
American Urologic Staging	Stanford (modification of UICC system)*	No tumor	Positive pelvic nodes only	Positive pelvic and para-aortic nodes†	No. of positive biopsies/total No. of biopsies	% positive biopsies
			Disease limited to the prostate			
A	T_0	1	0	0	0/1	
B	T_{1a}, T_{1b}	7	1	0	1/8	
	T_{1c}	8	0	2	2/10	
	T_{2a}	1	1	0	1/2	
	T_{2b}	9	3	0	3/12	
		26	5	2	7/33	21.2
			Extracapsular extension			
C	T_{3a}	1	0	0	0/1	
	T_{3b}	12	10	10	21/32	
		13	10	10	20/33	60.7
Totals		39 (59.1%)	15 (22.7%)	12 (18.2%)	27/66	40.9

*From ref No. 1.

†To date, all cases with positive para-aortic nodes have positive pelvic nodes as well.

TABLE 4.—Distribution of patients according to clinical stage and differentiation of primary tumor

No. of patients	Clinical stage: American Urologic Staging	Clinical stage: Stanford (modification of UICC system)*	Primary tumor differentiation: Well differentiated	Moderately differentiated	Poorly differentiated
1	A	T_0			1 (100%)
18	B	T_1	8 (44%)	8 (44%)	2 (11%)
14	B	T_2	3 (21%)	7 (50%)	4 (29%)
33	C	T_3	2 (6%)	12 (36%)	19 (58%)
66			13 (20%)	27 (41%)	26 (39%)

**From* ref No. 1.

TABLE 5.—Distribution of patients with nodal metastases according to stage and differentiation of primary tumor

No. of patients	Clinical stage: American Urologic Staging	Clinical stage: Stanford (modification of UICC system)*	Tumor differentiation†: Well differentiated	Moderately differentiated	Poorly differentiated	Patients with nodal metastases (%)
1	A	T_0			0/1	0
18	B	T_1	0/8	1/8	2/2	17
14	B	T_2	1/3	2/7	1/4	29
33	C	T_3	2/2 (100%)	5/12 (42%)	13/19 (69%)	61
66			3/13 (23%)	8/27 (30%)	16/26 (62%)	41

**From* ref No. 1.
†No. of patients with nodal metastases/No. in histopathologic group.

IV) by the method of Kempson and Levine (5). Patients with a mixture of grades are designated according to the least well-differentiated group. Only one patient had a grade IV tumor. As shown in table 4, there is a tendency for stages T_0, T_1, or T_2 tumors to be well or moderately differentiated, and for stage T_3 tumors to be moderately or poorly differentiated.

The distribution of patients with nodal metastases according to clinical stage and differentiation of the primary tumor is shown in table 5.

The difference in incidence of lymph node metastases is nearly significant (chi-square test = 3.71 for 1 degree of freedom) between the well-differentiated and poorly differentiated tumors, and is significant (chi-square = 4.23, $P \leq 0.05$) between the moderately differentiated and poorly differentiated tumors. Comparing all three grades, the incidence of lymph node metastasis increases significantly (chi-square with 2 degrees of freedom = 8.34, $0.05 > P > 0.01$) as the degree of differentiation decreases. Clearly, the 62% incidence of node metastases for the poorly differentiated (grades III and IV) tumors is the dominant factor.

Although the overall incidence of positive lymph node biopsies in patients with well-differentiated tumors was low, both patients with well-differentiated stage T_3 tumors had positive lymph node biopsies.

Treatment Results

Sixty-one patients have now completed radiation therapy. Thirty-seven of these patients had negative lymph node biopsies, while 24 had positive biopsies (14 patients with pelvic nodes, ten with both pelvic and para-aortic nodes) as shown in table 6. Of the 37 patients with negative lymph node biopsies, 32 (86.5%) are disease free. The median followup period for all of these 37 patients is 22 months (range, 1–50 months). Distant metastases were documented in the five treatment failures at 5, 17, 20, 37, and 46 months after completion of treatment. Obviously, the followup interval is still too short for a final analysis of survival rates.

Of the 14 patients with only positive pelvic lymph node biopsies, ten (71.5%) are disease free, but two of the ten have current abnormal studies suspicious for metastatic disease. The median followup interval for all 14 patients is 17 months (range, 1–50 months),

TABLE 6.—Current disease status of patients by distribution to treatment groups

Treatment group	No. of patients	No. of disease-free patients (%)
Negative lymph nodes at laparotomy		
Radiation therapy to prostate region only*	17	15
Radiation therapy to pelvic nodes with boost to prostate†	18	16
Radiation therapy to pelvic and para-aortic nodes with boost to prostate‡	2	1
	37	32 (86.5)
Positive pelvic lymph nodes at laparotomy		
Radiation therapy to pelvic nodes with boost to prostate	9	7
Radiation therapy to pelvic and para-aortic nodes with boost to prostate†	5	3
	14	10 (71.5)
Positive pelvic and para-aortic nodes at laparotomy		
Radiation therapy to pelvic and para-aortic nodes with boost to prostate	10	3 (30.0)
Totals	61	45 (73.8)

*1 patient randomized to prostate + pelvic node irradiation but treated only to prostate because of wound dehiscence.
†1 patient in each group excluded because he did not complete radiation therapy.
‡2 patients with negative node biopsies had reported tumor in retroperitoneal soft tissue (1 pelvis; 1 para-aortic) reinterpreted as organ of Zuckerkandl.

while the median followup for the ten disease-free patients is only 9 months (range, 1–39 months). The documentation of the intervals from treatment to local recurrence in one patient, and from treatment to the manifestation of distant metastases in three patients, ranged from 16 to 29 months (16, 24, 28, and 29 months) in the four patients who have failed treatment to date.

DISCUSSION

Because of the apparent inevitable development of bone metastases in patients with positive para-aortic nodes, the addition of prophylactic chemotherapy or skeletal irradiation is under consideration in this group of patients. It is obvious that radiation therapy alone is inadequate. In addition, patients with positive pelvic nodes will be observed carefully for the development of bone metastases inasmuch as it may be desirable or necessary to consider chemotherapy in this group if the incidence of metastasis escalates substantially with the passage of time. An interesting phenomenon appears to be emerging from this series. In order to appreciate this, it should be understood that at the beginning of this investigation approximately 6 years ago, the prognostic significance of the combination of lymphograms, histopathologic grade, elevated acid phosphatase in the absence of overt skeletal metastases, and surgical staging of lymph node involvement was unknown. It now appears possible that in the face of such comprehensive and accurate pretherapeutic evaluation, treatment options may become so well defined that the concept of the irradiation of clinically uninvolved lymph nodes will become irrelevant. This would pertain, of course, only in the presence of accurate mapping of potential metastatic sites. Clearly, additional data will be required to expand and confirm these preliminary observations.

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New Steroidal Alkylating Agents in Advanced Stage D Carcinoma of the Prostate [1]

Arnold Mittelman,* Raphael Catane, and Gerald P. Murphy [2]

SUMMARY

In a previous study, 50 patients with prostatic carcinoma were given continuous oral estramustine phosphate at a dose of 15 mg/kg/day. All patients had progressed on prior standard treatment. The objective and subjective response rates were 19% and 36% respectively. Seven of the 50 patients are still receiving treatment after 1–3 years. One patient who was given estramustine phosphate therapy for 1 year achieved a complete response, which included disappearance of osteoblastic metastasis. He still continues in unmaintained remission despite the fact that estramustine phosphate therapy was stopped because of gastrointestinal toxicity. No serious side effects related to the drug have been seen. Estramustine phosphate may be given safely for a prolonged period and has a place in the treatment of advanced prostatic cancer refractory to hormonal therapy. Twenty-one patients with stage D prostatic adenocarcinoma who failed hormonal therapy were treated with a combination of estramustine phosphate (600 mg/m²/day) plus prednimustine (Leo-1031) (15 mg/m²/day) in daily oral doses. Fifteen patients have been treated with prednimustine alone. The preliminary results of the combination therapy (after 2–9 months) are as follows: five patients (24%) had an objective response and nine patients (44%) had subjective improvement. Only five (24%) did not benefit from the drug and seven (33%) are stable. Of the 16 patients treated with prednimustine alone, one patient has had an unequivocal objective response and one experienced a considerable objective improvement. These preliminary results indicate the possible advantage of adding an alkylating agent (prednimustine) to estramustine phosphate in advanced prostatic carcinoma.

[Cancer Treat Rep 61:307–310, 1977]

Patients with carcinoma of the prostate who no longer respond to hormonal manipulation have a poor prognosis, with a median survival of 1 year (1). Patients with progressive and extensive stage D carcinoma of the prostate probably have a shorter median survival. Such patients frequently have extensive skeletal metastasis and replacement of bone marrow by prostatic cancer cells. Some of these patients have also received extensive pelvic radiation.

The need for effective chemotherapeutic agents whose primary toxicity is not myelosuppression is particularly felt in patients with advanced carcinoma of the prostate. Estramustine phosphate,[3] a conjugate of estradiol 17β with an alkylating agent (fig 1), and prednimustine (fig 2),[4] a conjugate of prednisolone and chlorambucil, seem to fulfill these requirements.

Estramustine phosphate has had clinical trials in Europe and most recently at Roswell Park Memorial Institute. We have demonstrated its clinical efficacy in patients refractory to conventional hor-

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[3]Estramustine phosphate: NSC-89199; CAS reg. No. 4891-15-0; estra-1,3,5(10)-triene-3,17β-diol, 3-[bis(2-chloroethyl)carbamate]17-(dihydrogen phosphate); Estracyt.

[4]Prednimustine: NSC-134087; CAS reg. No. 29069-24-7; pregna-1,4-diene-3,20-dione, 11β,17,21-trihydroxy-, 21-[4[p-[bis(2-chloroethyl)amino]phenyl]butyrate]; Leo-1031.

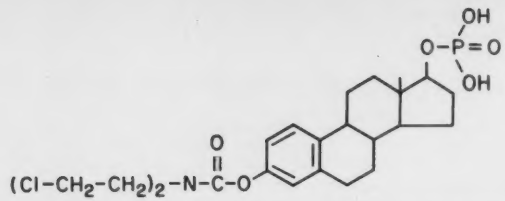

FIGURE 1.—Estramustine phosphate, a steroidal alkylating agent consisting of estradiol 17β with mechlorethamine linked to the C3 of the steroid.

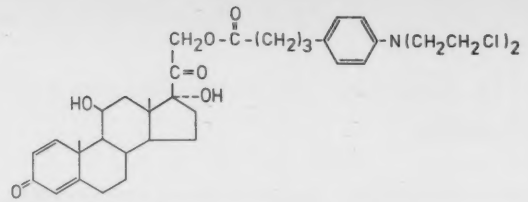

FIGURE 2.—Prednimustine, a steroidal alkylating agent consisting of prednisone with chlorambucil conjugated at the C21 position of the steroid.

monal therapy (2–4). Following the administration of estramustine phosphate, only the intact molecule has been demonstrated in the blood of man. No myelosuppression has been observed, the primary toxicity being gastrointestinal. It seemed logical to combine this agent with another cytotoxic agent, prednimustine, which has had moderate myelotoxicity (5–7).

The purpose of this study is to summarize our long-term experience with estramustine phosphate and to report our findings on the therapeutic usefulness of this agent combined with prednimustine for advanced carcinoma of the prostate.

PATIENT TREATMENT AND RESPONSE

Estramustine Phosphate

Our previous experience indicated a 19% objective response and a 36% subjective response in a group of 50 patients treated with this agent (8). This was achieved with a daily oral dose of 15 mg/kg. Of this group, seven patients are currently being followed. All have received estramustine phosphate for at least 1 year, some for > 3 years. Table 1 illustrates the type of response this group of patients experienced, including what must be a rare response to any therapy for prostatic carcinoma. This patient (No. 6) has experienced a complete regression of blastic skeletal metastases. He is in his second year of remission; his acid and alkaline phosphatase levels returned to normal and have remained so.

The safety of long-term estramustine phosphate therapy, in some instances for > 2 years, is confirmed by our present study. The only toxicity seen in this group was some nausea and vomiting and mild gynecomastia in two patients.

Estramustine Phosphate/Prednimustine Combination

Twenty-five patients with progressive stage D carcinoma of the prostate have been entered into

TABLE 1.—Characteristics of response to estramustine phosphate

Patient No.	Duration of estramustine phosphate therapy (mos)	Response		Side effects		
		Subjective	Objective	Mild	Moderate	Severe
1	37+	+	Stable	—		
2	36+	+	Elevated acid and alkaline phosphatase levels returned to normal	—	—	—
3	28	+	Stable	Edema	—	—
4	26+	+	Decrease in size of prostate and return of abnormal cystogram to normal	Gynecomastia	—	—
5	6	+	Stable	—	—	—
6	12	+	Disappearance of blastic metastases	—	Nausea, vomiting	—
7	12+	+	Stable	Gynecomastia	—	—

TABLE 2.—Characteristics of evaluable patients at start of therapy

No. of patients	21
Age in yrs (range)	65 (54–85)
No. with previous therapy	
Estrogen	21
Orchiectomy	12
Radiation	11
Chemotherapy	6
Yrs from diagnosis (range)	3.5 (0.5–10)

the study. Twenty-one are currently evaluable; four are too early in therapy to describe. Estramustine phosphate (600 mg/m²/day) and prednimustine (15 mg/m²/day) were given in divided oral doses. This regimen represents full therapeutic doses of both agents. Dose reduction of estramustine phosphate by 30% was instituted for severe gastrointestinal toxicity. In the event of hematologic toxicity (wbc count $< 3000/mm^3$ or platelet count $< 100,000/mm^3$), the prednimustine dose was reduced by 30%. In previous studies dosage schedules could be used with acceptable toxicity (7,9).

Table 2 describes some characteristics of patients with advanced stage D carcinoma of the prostate entered into this study. All patients had therapy with estrogens and half of these also received orchiectomies. Six patients had also received chemotherapeutic agents such as adriamycin, 5-fluorouracil, and cyclophosphamide. Table 3 shows the response to therapy in 21 evaluable patients. Of the five objective responders two had remissions of 4–6 months' duration. Three patients continue in remission for > 6 months. The majority of patients receiving the estramustine phosphate/prednimustine combination achieved some subjective benefit such as relief of bone pain, weight gain, and a sense of well being. Among responders some evidence of benefit occurred within the first month of therapy. The quality of response has been very good. Two patients with extensive pulmonary metastasis and elevated acid and alkaline phosphatase levels have had a complete regression of the pulmonary lesions and return of the abnormally elevated phosphatases to normal values. One of these patients had previously been treated with adriamycin and 5-fluorouracil with no effect.

TABLE 3.—Response to prednimustine and estramustine phosphate in advanced prostatic carcinoma

	No. of patients	%
Objective response*	5	24
Subjective response*	4	19
Total responses	9	43
Stable disease	7	33
Progression	5	24
Total	21	100

*National Prostatic Cancer Project criteria (9).

TABLE 4.—Toxicity from estramustine phosphate and prednimustine*

Toxic effect	No. of patients/ total	%
Gastrointestinal (nausea and vomiting)	4/21	19
Hematologic (blood count depression)	4/21	19
Edema	3/21	14

*No deaths were related to drug toxicity. In most cases, the side effects were eliminated by reducing the doses.

Table 4 lists the toxic reactions seen with this combination. The minor side effects seen in two patients were controlled with dose reductions. Two patients had intractable nausea and vomiting, requiring discontinuation of the program.

Prednimustine: Single-Agent Program

Sixteen patients have been entered into a study of prednimustine as single-agent therapy for advanced carcinoma of the prostate. All patients in this group were failures to hormonal therapy. Four of these patients were estramustine phosphate failures. The dosage schedule for this group was the same (15 mg/m²/day) that was used in the combination study. It is still too soon to draw conclusions concerning prednimustine alone in the therapy of advanced stage D carcinoma of the prostate. Table 5 lists our preliminary data. One objective and one subjective response have been observed.

DISCUSSION

Our study of long-term estramustine phosphate administration indicates its safety and therapeutic

TABLE 5.—Phase II study of oral prednimustine in advanced stage D carcinoma of the prostate

Patients entered	17
Patients too early to evaluate	8
Patients responding	
Objective	1
Subjective	1
Stable	5
Progression	2

value. It can therefore be considered for the treatment of earlier stages of prostatic carcinoma.

The series of studies presented here indicate the feasibility of combining specific steroidal alkylating agents for the therapy of advanced carcinoma of the prostate. This can be done using full therapeutic doses of each agent.

The 24% objective response rate and 43% subjective response rate reveal the potential usefulness of such combinations in the treatment of advanced disease. Patients in this group were failures not only to conventional hormonal therapy but to chemotherapy as well. This was achieved without significant toxicity. The use of prednimustine was based on experimental data indicating a superior therapeutic index compared to chlorambucil alone (10). This compound has been effective in the treatment of lymphoma and chronic lymphocytic leukemia patients who have relapsed on chlorambucil and prednisone (7,10). Our preliminary data indicate that prednimustine as a single agent is well tolerated by patients with advanced prostatic carcinoma and may also enhance response rates when used in combination with estramustine phosphate.

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Evaluation of Melphalan, ICRF-159, and Hydroxyurea in Metastatic Prostate Cancer: A Preliminary Report [1,2]

L. K. Kvols,* R. T. Eagan, and R. P. Myers [3]

Endocrine-unresponsive, metastatic prostate cancer is presently one of the malignant neoplasms most refractory to cytotoxic chemotherapy. A recent analysis of monochemotherapy for solid tumors revealed that only three of 29 "standard" antineoplastic agents had suggestions of activity against prostatic cancer (1). Two alkylating agents, cyclophosphamide and mechlorethamine, as well as 5-fluorouracil, were cited as the drugs with potential activity. Our previous clinical study involving patients with prostatic cancer revealed that adriamycin must be added to this list of active agents (2).

Searching for other single agents with activity in this refractory neoplasm, we have recently undertaken a prospective randomized clinical trial of three oral antineoplastic agents given individually to patients with endocrine-unresponsive, metastatic prostate cancer. The other major purpose of this study is to further evaluate the Ancillary Scoring System in assessing drug response. To date, this study is in its early stages and only preliminary data are presented.

MATERIALS AND METHODS

Twenty-one men, ranging in age from 64 to 81 years, with adenocarcinoma of the prostate have been entered in this study. All of the patients had failed bilateral orchiectomy as well as estrogen therapy, and none had had prior cytotoxic chemotherapy. All 21 patients had skeletal metastasis and in 14 this was the only evidence of metastatic disease.

The eligibility criteria for entry in this study are identical to those required in our previous investigational chemotherapy protocol (3). Prior to randomization, patients were stratified by three criteria: (*a*) age, < 65 or ≥ 65 years old; (*b*) Eastern Cooperative Oncology Group (ECOG) performance score, 0–1 or 2–3; and (*c*) predominant disease, bone only or other sites with or without bone. After stratification, patients were assigned by randomization to one of three oral treatment programs. Melphalan was given in a dose of 6 mg/m²/day for 5 consecutive days every 4 weeks; ICRF-159 was given in a dose of 850 mg/m²/day for 3 consecutive days every 4 weeks; and hydroxyurea was given in a dose of 3000 mg/m² every 3 days continuously. For melphalan and ICRF-159, dosages were adjusted to keep the leukocyte nadir between treatments > 2000 cells/mm³ and the platelet nadir $> 50{,}000$ platelets/mm³. When the leukocyte count was < 2000/mm³ and/or the platelet count was $< 75{,}000$/mm³ in patients receiving hydroxyurea, no treatment was given until leukocytes were > 3000/mm³ and platelets were $> 100{,}000$/mm³.

Response to therapy was evaluated by actual tumor measurements (excluding prostate size) and assessment of ancillary scoring factors (*see* table 1). If measurable disease was present, response was defined as a $\geq 50\%$ reduction in the product of the longest perpendicular diameters of the indicator lesion(s) or a $\geq 30\%$ reduction in the sum of the distance the liver edge extended below the costal margin(s) and xiphoid, and no new lesions. An objective progression required a $\geq 25\%$ increase in the indicator lesion(s) or liver or the appearance of any new lesions. Patients with skeletal metastases only were considered failures if new symptomatic lesions appeared on the bone scan or roentgenograms or the ancillary score was ≥ 15 overall, and they were considered stable if no change occurred in the bone scan or roentgenograms and the ancillary score was 10–14; responses were defined as no change or improvement in the bone scan and roentgenogram abnormalities and an ancillary score of < 10. Unless clear-cut progression was noted, response to therapy was assessed 10 weeks after the initiation of therapy. Leukocyte and platelet counts were checked once a week for 3 consecutive weeks after each course of therapy.

[1]Supported by contract CM-57044A from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-.

ICRF-159: NSC-129943; CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4'-propylenedi-, (±)-.

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1.

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TABLE 1.—Ancillary Scoring System (all changes relative to starting value for each drug)

Ancillary factors	Scores and changes*			
	0	1	2	4
Pain	None	Improved	Stable	Worse
Performance status (ECOG)	Asymptomatic	Improved	Stable	Worse
Weight (%)	Normal	Increased > 5	Stable (≤ 5)	Decreased > 5
Hemoglobin (g/100 ml)	Normal†	Increased > 1	Stable (≤ 1)	Decreased > 1
Total alkaline and acid phosphatase levels (%)	Normal	Decreased > 25	Stable (≤ 25)	Increased > 25

*Overall score of ≤ 9 considered a response; overall score of 10–14 considered stable; and overall score of > 15 considered progression.
†13 g/100 ml.

RESULTS

To date, a total of 21 patients have been entered in this study and 16 are evaluable for response to therapy. Five patients are new to the study and not yet evaluable. Eight evaluable patients have received melphalan: six of these are stable (2–6 months) and two experienced immediate disease progression. Five evaluable patients have received hydroxyurea: one is presently in partial remission, two are stable (2–4 months), and two experienced immediate progression. Only three of the patients treated with ICRF-159 are evaluable and all three are stable (3–5 months).

Toxicity has not been remarkable to date. All patients have experienced mild nausea with treatment but there has been no serious vomiting or stomatitis. No leukocyte nadirs < 2000 cells/mm^3 and no platelet nadirs < 75,000 platelets/mm^3 have been seen.

DISCUSSION

Successful combination chemotherapy for advanced prostatic cancer will be possible only when active single agents have been identified. The drug dosages and schedules we have been using in this study are quite tolerable. Our preliminary data from this study have been reported and to date only one patient has had what we would consider an objective response.

Using the Ancillary Scoring System in our previous study, all seven patients considered to have responded by more conventional criteria had ancillary scores of ≤ 9 (2). This system will undergo continued review in our current study.

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Preliminary Report on Combination Therapy for Advanced Prostatic Cancer

Claude Merrin [1,*]

SUMMARY

Fifteen patients with carcinoma of the prostate (ten, stage D; three, stage C; and two, stage B) were treated with the combination of radical retropubic prostatectomy, bilateral orchiectomy, estrogen therapy, and chemotherapy with 5-fluorouracil and cyclophosphamide. Fourteen patients have been clinically free of disease from 2 to 20 months. One patient died free of disease from myocardial infarction in the postoperative period. Lymphangiography was accurate in 14 of the 15 patients. Acid phosphatase levels in serum did not reflect with precision the extension of the tumor in seven of ten patients with stage D disease. The rationale of this therapeutic approach is discussed.

[Cancer Treat Rep 61:313–315, 1977]

The treatment of carcinoma of the prostate remains a subject of controversy. Different investigators recommend various therapeutic approaches (1–7). These approaches are very often contradictory and the results achieved are less than optimum. We have considered this problem and tried to establish a model of therapy based on the natural history of the disease. A preliminary report is presented.

METHODS

Fifteen patients were entered in this study from August 1974 to May 1976. Their ages ranged from 46 to 69 years with an average of 62.9 years. All the patients had prostatic carcinoma documented by tissue biopsy. None of the patients had bony metastases. There were 11 patients with clinical stage B and four with stage C disease as determined by rectal examination and general metastatic survey.

CLINICAL EVALUATION

The patients were evaluated by physical examination, iv pyelography, cystoscopy, perineal needle biopsy, chest roentgenogram, bone survey, bone scan, liver scan, electrocardiogram, bilateral pedal lymphangiography, bone marrow aspiration, and routine biochemical and hematologic determinations.

All the patients had a radical retropubic prostatectomy with bilateral orchiectomy. Two patients had retroperitoneal lymphadenectomy up to the level of the renal vessels. Two patients had symphysiectomies. After surgery all the patients were started on diethylstilbestrol (1 mg daily) and chemotherapy. They received 5-fluorouracil (5-FU) (8 mg/kg of body weight daily for 5 days every month) and concurrently cyclophosphamide (4 mg/kg of body weight for 5 days every month).[2]

RESULTS

Therapy

Fourteen patients are presently alive and clinically free of disease from 2 to 20 months. One patient died from myocardial infarction in the postoperative period; he was free of tumor at autopsy. The average followup to date in all the patients is 7 months.

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[2] 5-Fluorouracil: NSC-19893; CAS reg. No. 51–21–8.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055–19–2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

TABLE 1.—Correlation of stages by lymphangiography and pathology in patients initially considered as having stage B and C disease

Initial clinical stage	No. of patients	Stages (No. of patients) based on— Lymphangiography	Pathology
B	11	B (4)	B (2)
		C (0)	C (2)
		D_1 (6)	D_1 (6)
		D_2 (1)	D_2 (1)
C	4	D_1 (3)	C (1)
		D_2 (1)	D_1 (2)
			D_2 (1)

Lymphangiography

Of the 11 patients considered to have stage B disease on rectal examination, six were stage D_1 and one was D_2 by lymphangiography. Histologic analysis of the specimens confirmed the lymphangiographic findings. Lymphangiograms were negative for the four remaining patients. On pathologic analysis two patients had stage C and two had stage B disease (table 1).

Of the four patients considered to have stage C disease initially, three had stage D_1 and one had stage D_2 by lymphangiography. By pathology, two patients had D_1, one had D_2, and one had C. There was one false positive lymphangiogram (table 1).

Acid Phosphatase

The acid phosphatase was elevated in three patients with stage D disease. It was within normal limits in seven patients with stage D, three patients with stage C, and two patients with stage B (table 2).

COMPLICATIONS

Surgery

All the patients tolerated the surgery well. Leakage of the vesico-urethral anastomosis was present in four patients. In these four patients an indwelling Foley catheter was left in the bladder for an additional period of time. Healing of the urethrovesical anastomosis occurred between 2 and 4 weeks.

Incontinence and stress incontinence were present in all the patients initially. Continence was regained in 2–8 weeks in 14 patients. One patient remained incontinent.

TABLE 2.—Correlation between acid phosphatase levels in serum and extension of disease

Pathologic stage	No. of patients	Acid phosphatase level
B	2	Normal
C	3	Normal
D	7	Normal
	3	Elevated

Cardiovascular

One patient died in the post-operative period from myocardial infarction. Two patients, while receiving estrogen therapy, developed cardiovascular complications (one myocardial infarction and one pulmonary embolism). Estrogen therapy was immediately discontinued in these patients.

Chemotherapy

Different degrees of nausea and/or vomiting were present in all the patients after administration of the 5-FU and cyclophosphamide. This toxicity was transient and responded well to antiemetics. Mild-to-moderate myelosuppression (wbcs between 2500 and 3800/mm^3) was seen in ten patients. This occurred between 1 and 3 weeks after drug administration and did not require any therapeutic measures.

DISCUSSION

Carcinoma of the prostate appears to be composed of a heterogeneous population of cells. These cells have different evolutive potentials and respond to different stimuli. They can theoretically be divided into three groups: (*a*) cells with androgen receptors (8); (*b*) cells with androgen and estrogen receptors (8,9); and (*c*) cells without hormonal receptors (10).

In the past, treatments have been mostly directed against only one of the cellular groups of this tumor (1–6). Therefore, it is not surprising to observe that cure has rarely been achieved. To palliate this problem, we have intended to establish a model of therapy which takes into consideration all the possible routes of therapeutic resistance of the tumor. For this we have considered (*a*) the basic kinetics of solid tumors, and (*b*) the particular characteristics of carcinoma of the prostate.

It is known experimentally and clinically that there is an inverse relationship between the effectiveness of chemotherapy and the mass of a tumor (11). The larger the tumor mass, the less effective the chemotherapy will be. As a consequence, an initial decrease in the tumor burden becomes an

essential preparatory step in securing the success of any future treatment with chemotherapy (12). This goal can be achieved by local and/or regional excision of the tumor. Therefore, our treatment starts by a radical retropubic prostatectomy in order to decrease the tumor burden to a minimum.

The remaining tumor is composed of hormonal responsive and non-hormonal responsive cells. The hormonal responsive cells are suppressed by the conjunction of orchiectomy and estrogen administration. The orchiectomy, by decreasing the androgen production, suppresses their stimulatory cellular effects. Estrogen administration, by acting directly at the cellular level as well as indirectly through the hypophysis, further decreases the vitality of these cells. The non-hormonal responsive cells are then attacked by combination chemotherapy with cyclophosphamide and 5-FU. This combination is more effective than the single drugs given alone. It is well tolerated by the patients.

These preliminary results are not in contradiction with the initial working hypothesis. Presently, all the patients are clinically free of disease.

Another interesting aspect of this study is the lack of clinical symptomatology observed in most of the patients, even when they already had advanced disease. Ten of the 15 patients had silent lymphatic metastases.

It has been traditionally accepted that carcinoma of the prostate remains latent for a long period of time. This opinion has been based on the long clinical silence observed in many cases (13). The autopsy findings in men with occult carcinoma of the prostate who died from other causes have also substantiated this thinking (14). Clinical silence has been widely interpreted as a lack of biologic activity by many practicing clinicians. The findings in our limited group of patients appear to be in contradiction with this concept. Carcinoma of the prostate may lack clinical expression for a long period of time, but is highly active biologically from its initiation. The early metastases are silent and limited to the regional lymphatics. The bone metastases occur at a later date.

In the past, most clinical centers did not study the possible lymphatic involvement by the tumor. Lymphangiography has long been considered inadequate for this purpose. In contradiction with this position, the experience in our group of patients reveals a correct lymphangiographic reading in 14 of 15 patients. (One patient was a false positive because of a lipoid granulomatous reaction to the ethiodol.) These results in this small group of patients are enough to emphasize the importance of lymphangiography for the correct clinical staging of patients with carcinoma of the prostate.

Of the ten patients with lymphatic metastases, only three had elevated acid phosphatase levels and the other seven had normal acid phosphatase levels in the serum. The findings in these patients seem to disprove the widespread belief that a normal acid phosphatase level may rule out metastases in an apparently local disease.

The limited number of patients and the short followup time in this study do not permit definitive conclusions about the value of this combined therapeutic approach for the treatment of carcinoma of the prostate. The results reported here are preliminary. Observations of more patients with a longer followup will be the subject of future communications.

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National Randomized Study of Chemotherapeutic Agents in Advanced Prostatic Carcinoma: A Progress Report [1]

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SUMMARY

In the 36 months since its inception, the National Prostatic Cancer Project treatment subgroup has randomly assigned over 360 patients with progressive advanced prostatic cancer who were no longer responsive to endocrine manipulation to either one of four different clinical studies. The initial study demonstrated a clear superiority for 5-fluorouracil (5-FU) and cyclophosphamide over continued conventional therapy. Beneficial responses were documented and are associated with increased survival rates and relief from pain and other symptoms. A proportionately larger number of patients obtained clinical benefit (stable and partial regression) on cyclophosphamide than on standard or 5-FU therapy. The criteria for evaluation of patients are supported by the survival data, ie, responders have survived for a longer period of time than those patients who continued in progression. Preliminary data from the subsequent protocols have documented a 30% response (stable and partial regression) in patients receiving oral estramustine phosphate and definite responses in patients treated with DTIC. Too few patients have been treated with Leo 1031 to offer total response rates at this time, although the early results are promising. These clinical studies have firmly established a place for chemotherapy in the management of prostatic cancer. New trials will introduce single- and multiple-drug chemotherapy at earlier phases of the clinical course of prostatic cancer patients.

[Cancer Treat Rep 61:317–323, 1977]

In July 1973, the treatment subgroup of the National Prostatic Cancer Project (NPCP) composed of five different centers in the United States initiated the first of four randomized trials comparing the efficacy of a variety of chemotherapeutic agents in the treatment of patients with metastatic carcinoma who had relapsed following hormonal and/or other types of adequate conventional (standard) treatment. The initial protocol randomizing 125 endocrine-resistant prostatic cancer patients to treatment with either continued standard therapy, 5-fluorouracil (5-FU), or cyclophosphamide was initially described in 1975 (1–3). The objective response rates for initial therapy (stable plus partial regression) were 36% for 5-FU and 46% for cyclophosphamide, and the response rate for standard therapy (stable only) was 19%. Followup documentation that the objective and subjective response rates are also associated with increased survival was also provided (4). This initial study clearly demonstrated the beneficial objective and subjective results which could be obtained from the use of chemotherapeutic agents in the treatment of relapsing advanced pros-

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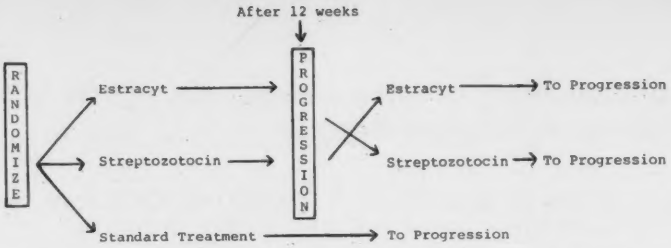

FIGURE 1.—NPCP-200, treatment scheme: estramustine phosphate (Estracyt) (600 mg/m²/day orally in 3 divided doses), streptozotocin (500 mg/m²/day iv × 5 days q6wks), or standard treatment. A crossover will be done at progression unless death occurs.

tatic carcinoma. Encouraged by those results, further clinical trials of additional agents were instituted and this report describes the current progress.

MATERIALS AND METHODS

Chemotherapeutic agents selected for study had previously demonstrated antineoplastic activity in other malignancies, had demonstrated effectiveness in animal model systems (5,6), and/or had demonstrated clinical benefits in prostatic cancer patients.[9]

All patients entered in the study have had far-advanced histologically confirmed clinical stage D disease which was progressing and had failed to respond to, or had relapsed following, conventional hormonal or ablative therapy. Treatment responses were evaluated on the basis of previously defined criteria (2,7–9). Patients who had received > 2000 rads of irradiation therapy to the pelvis were initially randomized by the NPCP Statistical Office to receive either oral estramustine phosphate, streptozotocin, or standard therapy (NPCP-200, fig 1). Patient entry for this protocol began on July 1, 1974, and was completed on April 2, 1976, with a total of 125 patients entered. Those patients who have not received extensive irradiation to the pelvis (> 2000 rads) are currently being randomized to receive either procarbazine, DTIC, or cyclophosphamide (NPCP-300, fig 2). Patient entry in this protocol started on April 1, 1975. Currently, patients who have previously received extensive pelvic irradiation are being randomly assigned treatment with a combination of estramustine phosphate and Leo 1031 or Leo 1031 alone (NPCP-400, fig 3). Patient entry for the latter protocol was initiated on April 2, 1976. For all protocols the data in this report have been evaluated through May 28, 1976.

[9]Catane R, Kaufman J, Mittelman A, et al. Combined therapy of advanced prostatic carcinoma with estramustine and prednimustine. Submitted to the *Journal of Urology*.

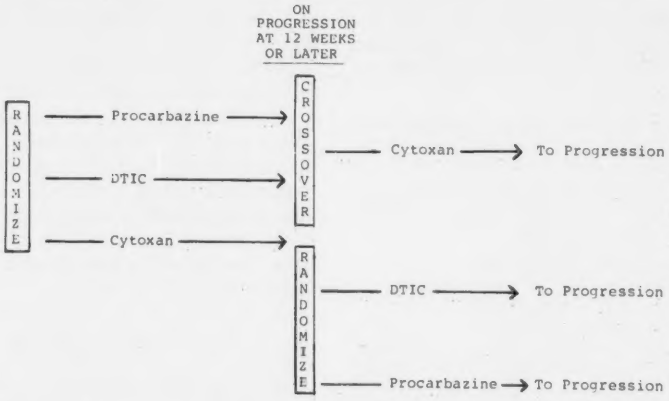

FIGURE 2.—NPCP-300, treatment scheme: procarbazine (100 mg/m²/day orally, Days 1–22, 3 wks' rest; therapy again on Days 44–65, 3 wks' rest, etc), DTIC (200 mg/m² iv, Days 1–5, 21 days' rest, then repeat cycle), or cyclophosphamide (Cytoxan) (1 g/m² iv q3wks). A crossover will be done at progression unless death occurs.

TABLE 1.—NPCP-200: comparison of estramustine phosphate, streptozotocin, and standard treatment—status of patients entered on study*

	Treatment			
	Standard	Estramustine phosphate	Streptozotocin	Totals
Patients on study > 3 wks	19	41	35	95
Patients off study within 3 wks	4	5	6	15
Protocol violations	0	2	0	2
Patients recently randomized	2	6	5	13
Totals	25	54	46	125

*Patients were accrued from July 1, 1974, to April 2, 1976, and evaluated through May 28, 1976.

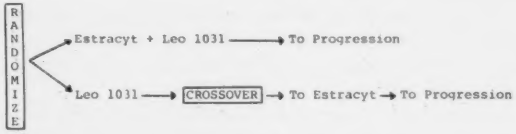

FIGURE 3.—NPCP-400, treatment scheme: estramustine phosphate (Estracyt) (600 mg/m²/day orally in 3 divided doses) + Leo 1031 (30 mg/day orally in 3 divided doses for 6 of every 7 days), or Leo 1031 (30 mg/day orally in 3 divided doses for 6 of every 7 days).

RESULTS

NPCP-200: Comparison of Estramustine Phosphate, Streptozotocin, and Standard Treatment

During the 21-month period of patient accrual, 125 patients were entered in the study as shown in table 1. Only two protocol violations occurred; one was a patient who was entered without previous irradiation treatment and the other was a patient whose life expectancy was < 90 days.

Analysis of the prognostic factors on entrance in the study has revealed no significant differences for each treatment arm of the study with regard to: (*a*) age distribution; (*b*) initial performance status; (*c*) elevation of acid phosphatase determination; (*d*) serum hemoglobin level; and (*e*) presence of chronic disease. These findings suggest that no single treatment arm received more than its share of favorable or unfavorable patients.

The objective responses to treatment are shown in table 2. The response rates (stable plus partial regression) were 16% in the standard group (stable only), 30% in the estramustine phosphate group (stable and partial regression), and 29% in the streptozotocin group (stable only). Partial regressions were obtained with only estramustine phosphate (two patients).

Toxicity studies revealed no significant differences between the chemotherapy and standard treatment arms of the study with regard to the development of leukopenia, thrombocytopenia, and alterations in renal function as measured by serum creatinine levels. The incidence of at least a moder-

TABLE 2.—NPCP-200: comparison of estramustine phosphate, streptozotocin, and standard treatment—objective response to initial therapy*

	Response			
Treatment	Partial regression	Stable	Progression	Totals
Standard	0	3	16	19
Estramustine phosphate	2	10	28	40
Streptozotocin	0	10	25	35
Totals	2	23	69	94†

*Patients were accrued from July 1, 1974, to April 2, 1976, and evaluated through May 28, 1976.

†1 estramustine phosphate patient was crossed over before 3 wks and is not included in this table.

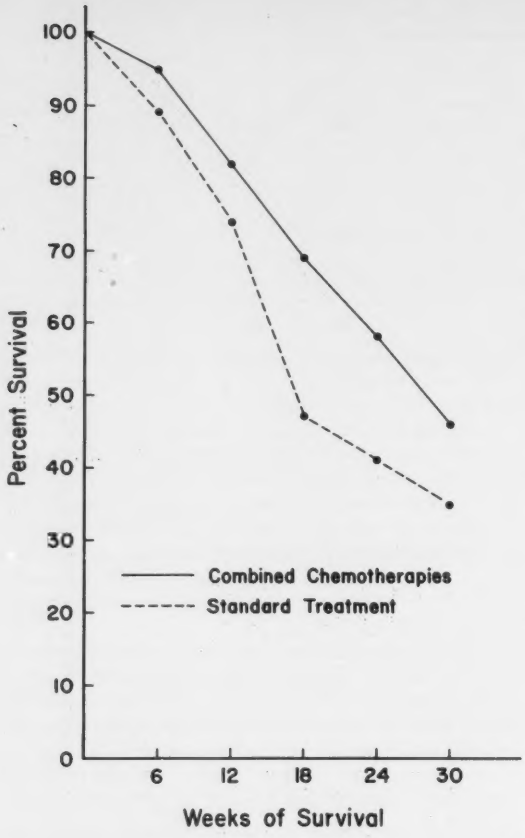

FIGURE 4.—NPCP-200: comparison between the survival rate for patients receiving chemotherapy (estramustine phosphate + streptozotocin) and standard treatment.

ate degree of nausea and vomiting attributable to disease and/or treatment was 37%, 49%, and 37% for patients on standard, estramustine phosphate, and streptozotocin therapy respectively.

The survival experience for these patients is displayed in figure 4. Even though the survival rate at 18 weeks is 22 percentage points better in the chemotherapy group than in the standard group, this difference is not as yet totally significant.

Estimates of the average survival time (computed by using the maximum likelihood estimator under the exponential model allowing for censorship) for the standard, estramustine phosphate, and streptozotocin treatment groups are 32.9, 45.2 and 57.6 weeks respectively. However, these estimates are not yet complete because of the number of patients who have not died.

Estimates of the average length of response, using the response times to date, in the standard, estramustine phosphate, and streptozotocin groups are 13.7, 49.9, and 41.8 weeks respectively.

It should be emphasized that although these data are still under periodic review, and should be considered preliminary, our early tabulations would seem to indicate that estramustine phosphate is the superior drug, producing favorable responses (stable plus partial regression) in approximately 30% of the patients treated.

NPCP-300: Comparison of Procarbazine, DTIC, and Cyclophosphamide

Table 3 illustrates the status of the 86 patients who have been entered in this study during its first 14 months of activation. Objective responses based on the 49 patients listed in the study after ≥ 3 weeks of treatment are shown in table 4. The re-

TABLE 3.—NPCP-300: comparison of procarbazine, DTIC, and cyclophosphamide—status of patients entered on study*

	Treatment			
	Cyclophos-phamide	DTIC	Procarbazine	Totals
Patients on study > 3 wks	11	21	17	49
Patients off study within 3 wks	1	4	3	8
Patients recently randomized	7	8	14	29
Totals	19	33	34	86

*Patients were accrued from April 1, 1975, through May 28, 1976. Randomization was agreed on beforehand to be 2 of 5 patients receiving procarbazine, 2 of 5 patients receiving DTIC, and 1 of 5 patients receiving cyclophosphamide.

TABLE 4.—NPCP-300: comparison of procarbazine, DTIC, and cyclophosphamide—objective response to initial therapy*

Treatment	Response			
	Partial regression	Stable	Progression	Totals
Cyclophosphamide	1	2	8	11
DTIC	2	8	11	21
Procarbazine	0	5	12	17
Totals	3	15	31	49

*Patients were accrued from April 1, 1975, and evaluated through May 28, 1976.

TABLE 5.—NPCP-300: comparison of procarbazine, DTIC, and cyclophosphamide—effect of treatment on subjective response parameters*

	Treatment		
	Cyclophosphamide	DTIC	Procarbazine
	No. of responses/No. of patients (%)	No. of responses/No. of patients (%)	No. of responses/No. of patients (%)
Pain relief	3/11(27)	6/21(29)	3/17(18)
Performance status improvement	5/11(45)	7/21(33)	3/17(18)
Weight gain	0/6(0)	0/13(0)	2/11(18)

*Patients were accrued from April 1, 1975, through May 28, 1976. Responses are noted for those patients who need improvement in subjective parameters.

sponse rates for cyclophosphamide, DTIC, and procarbazine, considering both stable disease as well as partial regressions as responses, are 27%, 48%, and 29% respectively. The effect of treatment on subjective response parameters is presented in table 5.

To date, toxicity has been minimal. Nausea and vomiting of at least a moderate degree has been reported in 43% of the patients. Leukopenia (wbc count $< 4000/mm^3$) has developed in 25% of the patients but was life threatening in only one. Thrombocytopenia has occurred principally in patients receiving DTIC (five of 21 patients) and in one of 17 patients receiving procarbazine.

NPCP-400: Comparison of Estramustine Phosphate Plus Leo 1031 Versus Leo 1031 Alone

Insufficient time has elapsed with too few patients (26) entered in this study to warrant further discussion at this time.

DISCUSSION

The results obtained thus far, although encouraging, are still of a preliminary nature. With 13 patients recently randomized to protocol NPCP-200, and a large proportion of the remaining patients still alive and under initial therapy, it is still too early to predict final responses or survival for the latter study as well as for the subsequently initiated chemotherapy trials. Catane et al[10] have reported a 19% objective response rate for clinical stage D prostatic cancer patients receiving estramustine phosphate alone, and a 24% objective response rate for similar patients receiving combined estramustine phosphate plus Leo 1031 therapy. All of the patients in NPCP-200 and -400 had prior radiotherapy of > 2000 rads, and when entered in the study (NPCP-200) had a significantly poorer performance status than had previously been observed for patients entered in NPCP-100. From previous experience with the latter protocol, even patients who had received < 2000 rads of prior irradiation responded poorly to chemotherapy. Whether this factor combined with a poorer on-study performance status will affect ultimate survival and response rate remains to be seen.

Although a variety of drugs had been used in the past for treatment of metastatic adenocarcinoma of the prostate (10),[11] it was not until the NPCP began

[10] *See* footnote 9.

[11] Slavik M, and Carter SK. Single agents in prostatic cancer—a quick review. Personal communication to the National Prostatic Cancer Project.

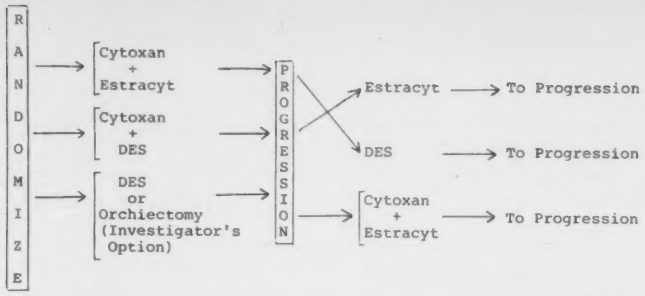

FIGURE 5.—NPCP-500, treatment scheme: cyclophosphamide (Cytoxan) (1 g/m² iv q3wks) + estramustine phosphate (Estracyt) (600 mg/m²/day orally in 3 divided doses), cyclophosphamide (1 g/m² iv q3wks) + diethylstilbestrol (DES) (1 mg tid orally), *or* diethylstilbestrol (1 mg tid orally), *or* orchiectomy. Patients whose disease progresses after the initial 12 wks of therapy will be crossed over.

its randomized clinical trials that the efficacy of chemotherapeutic agents was first demonstrated in this disease (2). Although the drugs that are being given today do not in any way represent a panacea for the treatment of this disease, current response rates ranging upward to 48% justify, as Murphy (11) suggested several years ago, consideration for their use as adjunctive therapy in earlier stages of the disease. Consequently, the NPCP treatment subgroup had, as of June 1976, initiated additional protocols calling for earlier patient exposure to combination chemohormonal therapy (NPCP-500 and -600, figs 5 and 6). These new studies will carefully evaluate the use of adjuvant chemotherapy with hormonal manipulation as a means for prolonging the nonprogressing interval in patients with recently diagnosed metastatic disease.

In the past, the criteria for measuring response to chemotherapy of prostatic cancer in its advanced stages were limited (1), but in-depth evaluation of our currently employed response parameters (7–9) has supported their objectivity (4). These findings, coupled with available test systems for chemotherapeutic agents against prostatic cancer (5,6) and the recent developments in animal tumor model systems, suggest that the suitability for so-called empiric clinical studies is limited. Although the search for better drugs continues, chemotherapy alone, in combination, or in the form of multiple agents has today achieved a definite place in the treatment of prostatic carcinoma.

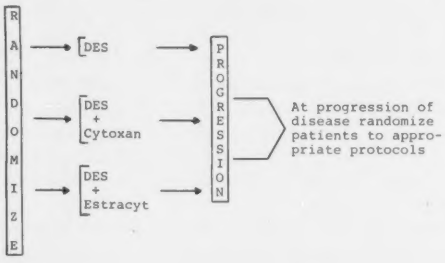

FIGURE 6.—NPCP-600, treatment scheme: diethylstilbestrol (DES) (1 mg tid orally), diethylstilbestrol (1 mg tid orally) + cyclophosphamide (Cytoxan) (1 g/m² iv q3wks), *or* diethylstilbestrol (1 mg tid orally) + estramustine phosphate (Estracyt) (600 mg/m²/day orally in 3 divided doses).

LIST OF COMPOUNDS

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
Diethylstilbestrol: NSC-3070; 4,4'-stilbenediol, α,α'-diethyl-; stilbestrol
DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-
Estramustine phosphate: NSC-89199; CAS reg. No. 4891-15-0; estra-1,3,5(10)-triene-3,17β-diol, 3-[bis(2-chloroethyl)-carbamate] 17-(dihydrogen phosphate); Estracyt
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8
Leo 1031: NSC-134087; CAS reg. No. 29069-24-7; pregna-1,4-diene-3,20-dione, 11β,17,21-trihydroxy-, 21-[4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyrate]
Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride
Streptozotocin: NSC-85998

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Comparative Trial of Adriamycin and 5-Fluorouracil in Advanced Prostatic Cancer—Progress Report [1]

William D. DeWys,* Madeline Bauer, Jacob Colsky, Richard A. Cooper, Richard Creech, and Paul P. Carbone [2]

Cytotoxic chemotherapy for adenocarcinoma of the prostate has received limited systematic evaluation. A number of studies are delineating the activity of single drugs in this disease, while others are beginning to evaluate the effects of various combinations. The predominant pattern of metastatic spread of prostatic cancer is the development of diffuse blastic bony metastases which makes quantitative study of this disease somewhat difficult. Only a limited number of patients develop objectively measurable disease which can be followed for response to chemotherapy and such measurable disease often develops very late in the course of the patient's disease. Since these patients with advanced disease often have significant systemic effects of their malignancy, they are not ideal candidates for chemotherapy.

This paper is a progress report of the comparative trial of 5-fluorouracil and adriamycin which is currently in progress in the Eastern Cooperative Oncology Group (ECOG). Eighty-two patients have been entered since this trial was activated in October 1973. This report will be based on an analysis of the 60 patients who have completed treatment and whose case records are available.

MATERIALS AND METHODS

Patients with histologically confirmed adenocarcinoma of the prostate not amenable to curative surgery or radiotherapy were eligible for this study if they met the following requirements:

(*a*) either measurable tumor (including soft tissue, lung nodules, well-defined retroperitoneal lymph nodes [defects on intravenous pyelogram], or hepatic enlargement > 5 cm below the costal margin) is present or two or more acid phosphatase determinations were elevated;

(*b*) adequate hepatic, renal, and marrow function (bilirubin ≤ 1.5 mg/100 ml, SGOT ≤ 80 IU, BUN ≤ 30 mg/100 ml, creatinine ≤ 1.5 mg/100 ml, wbc count $\geq 4000/mm^3$, and platelet count $\geq 100{,}000/mm^3$);

(*c*) performance status of ≤ 3 on the ECOG scale;

(*d*) no prior treatment with the drugs to be studied;

(*e*) no prior history of congestive heart disease; and

(*f*) at least 6 weeks had elapsed since ablative surgery or initiation of additive hormonal therapy and at least 2 weeks had elapsed since cessation of additive hormonal therapy. Patients receiving estrogen therapy could continue to receive this agent.

At the time of randomization, patients were stratified on the basis of ECOG performance status (0–1 = normal or slightly limited activity; 2–3 = partially bedridden), disease determination (measurable versus nonmeasurable), and extent of previous radiotherapy (minimal versus extensive). Radiation extent was defined as the product of radiation dose and fraction of marrow volume irradiated (1). A radiation extent score of ≥ 1000 was considered

[1] Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.

[2] This study was done by the Eastern Cooperative Oncology Group (Paul P. Carbone, chairman) and was supported by Public Health Service grants from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare. The following institutions participated in the study: Northwestern University Medical School, Chicago, Ill (*W. D. DeWys*, CA-17145); State University of New York, Buffalo (*M. Bauer*, CA-12721); Jackson Memorial Hospital, Miami, Fla (*J. Colsky*, CA-02822); University of Pennsylvania Hospital, Philadelphia (*R. A. Cooper*, CA-15488); The American Oncologic Hospital, Philadelphia, Pa (*R. Creech*, CA-18281); and University of Wisconsin Medical School, Madison (*P. P. Carbone*, CM-33745). Other participating institutions were: Albany Medical College of Union University (CA-06594), Boston University Medical Center (CA-11109); Chicago Medical Center at Mt. Sinai Hospital Medical Center (CA-14144); Georgetown University Medical School (CA-02824); Hahnemann Medical College (CA-13611); NCI; Centre Hospitalier Universitaire Lariboisier (Paris); University of Rochester Cancer Center (CA-11083); Rush-Presbyterian-St. Luke's Medical Center (CA-10948); University of Pretoria (South Africa); Lemuel Shattuck Hospital, Tufts University (CA-07190); Medical College of Virginia (CA-10572); Albert Einstein College of Medicine (CA-14958); Baystate Medical Center; Brookdale Hospital Center; University of Manitoba Medical School; Mount Sinai Medical Center; and University of Florida.

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extensive. Patients were randomly assigned by a central telephone randomization desk to receive either adriamycin or 5-fluorouracil.

Patients who had received minimal or no previous radiotherapy received either 5-fluorouracil (600 mg/m²/week iv) or adriamycin (60 mg/m² iv every 3 weeks). Patients who had received extensive prior radiotherapy received reduced doses of 5-fluorouracil (400 mg/m²/week iv) or adriamycin (40 mg/m² iv every 3 weeks). If after 6 weeks at reduced doses these patients had not experienced significant hematologic toxicity, the dose of either drug was increased by 50%.

Objective tumor response, for patients entering with measurable disease, was defined as follows: *complete response*—complete disappearance of all measurable disease with no new lesions developing; *partial response*—a 50% reduction in the sum of the products of the horizontal and vertical tumor diameters; *no change*—tumor shrinkage not fulfilling the partial response criteria or an increase of ≤ 25% in the sum of the products of the horizontal and vertical tumor diameters; and *progression*—an increase of > 25% in the sum of the products of the tumor diameters or the development of new lesions.

For patients without measurable disease but with initially elevated acid phosphatase levels, a reduction of ≥ 50% from the pre-treatment acid phosphatase level was considered to be a response. A decrease of < 50% or an increase of < 50% was considered no change, and an increase of ≥ 50% or the development of new lesions was considered disease progression.

RESULTS

Table 1 shows the objective tumor response by initial treatment for the 28 patients who entered with measurable disease. The treatments are coded since this study is still in progress. Of the 16 patients who initially received treatment 1, there was one partial response (6%) and no complete responses; of the 12 patients who received treatment 2, two had partial responses (17%) and one had a complete response (8%).

TABLE 1.—Objective tumor response to initial treatment (patients with measurable disease)

	Treatment	
Response	1*	2*
Complete	0 (0)	1 (8)
Partial	1 (6)	2 (17)
No change	6 (38)	3 (25)
Progression	9 (56)	6 (50)
Totals	16 (100)	12 (100)

*No. of patients (%).

Since complete records of acid phosphatase levels were not available for more than half of the 32 patients who entered the study without measurable disease, the main comparisons of treatment effects will be based on survival times calculated from the start of protocol therapy. The prognostic value of selected variables on survival is presented in table 2. Forty-three of the 60 patients in this analysis had a poor performance status (2 or 3). These patients had a shorter median survival than the patients with a good performance status (0 or 1). Patients who had received extensive prior radiotherapy had a shorter median survival than patients receiving minimal or no prior radiotherapy. The median survival for patients with objectively measurable disease is comparable to the median survival for patients whose only evidence of disease was elevated acid phosphatase.

The median survival for all patients initially treated with treatment 1 was 17 weeks compared to 26 weeks for patients initially treated with treatment 2. The relationship between treatment and each prognostic variable is presented in tables 3–5. The median survival for patients with good performance status is comparable for both treatments, but the number of patients in these subgroups is quite small. For patients with poor performance status,

TABLE 2.—Median survival by prognostic factor

Prognostic factor	Level*	Median survival (wks)	No. of deaths/ No. of patients
Performance status	0–1	29	8/17
	2–3	19	33/43
Prior radiotherapy	Minimal	23	27/41
	Extensive	16	14/18
Disease determination	Measurable	18	22/28
	Nonmeasurable	22	19/32

**See* Materials and Methods for definitions.

TABLE 3.—Median survival for treatment by performance status

Treatment	Performance status		
	0–1	2–3	Totals
1	25* (5/11)	11 (17/22)	17 (22/33)
2	24 (3/6)	21 (16/21)	26 (19/27)

*Wks (No. of patients dead/total No. of patients).

TABLE 4.—Median survival for treatment by prior radiotherapy

Treatment	Extent of prior radiotherapy	
	Minimal	Extensive
1	19* (15/22)	12 (7/10)
2	29 (12/19)	16 (7/8)

*Wks (No. of patients dead/total No. of patients).

TABLE 5.—Median survival for treatment by disease determination

Treatment	Disease determination	
	Measurable	Nonmeasurable
1	10* (12/16)	21 (10/17)
2	25 (10/12)	21 (9/15)

*Wks (No. of patients dead/total No. of patients).

the median survival on treatment 2 was approximately double the median survival for treatment 1, however, this difference is not statistically significant.

Table 4 gives the median survival for each treatment for patients with minimal and extensive prior radiotherapy. Patients who received treatment 2 tend to survive longer than patients who received treatment 1, regardless of the extent of prior radiotherapy. This difference between treatments is even more pronounced for patients who received minimal or no prior radiotherapy.

In table 5 the median survival for each treatment is compared within each category of disease determination. The medians for each treatment are comparable for patients with nonmeasurable disease, however, for patients with measurable disease, the median for treatment 2 is more than double the median for treatment 1.

The pattern of toxicity reported for the two treatments is shown in table 6. Most of the toxicity

TABLE 6.—Toxicity data

Type	No. of patients with degree of toxicity—			
	Mild	Moderate	Severe	Life threatening/ lethal
5-Fluorouracil				
Hematologic	14	8	6	0
Infection	0	0	0	0
Vomiting and nausea	7	1	0	0
Diarrhea	0	0	1	0
Other gastrointestinal	1	1	0	0
Bleeding	0	0	0	0
Skin and mucous membrane	2	0	0	0
Most severe toxicity	14	8	6	0
Adriamycin				
Hematologic	11	3	5	1*
Infection	0	1	0	1
Vomiting and nausea	9	7	2	0
Diarrhea	0	1	0	0
Other gastrointestinal	2	1	0	0
Bleeding	1	0	0	1*
Skin and mucous membrane	1	1	0	0
Cardiac	1	0	0	0
Most severe toxicity	10	9	7	2

*Disseminated intravascular coagulation.

experienced by patients receiving 5-fluorouracil was of mild or moderate degree; no one experienced life-threatening toxicity. Toxicity reported for patients receiving adriamycin was more severe, including two episodes of life-threatening disseminated intravascular coagulation experienced by one patient and one case of lethal infection experienced by another.

DISCUSSION

Although these results are preliminary, we are beginning to see a difference between the response to the two drugs under study. Treatment 2 has given a better response rate and a better overall median survival, but neither of these differences are statistically significant. The improvement in median survival was especially visible in patients with poor performance status, patients with limited prior radiotherapy, and patients who entered the study with measurable disease. The improvement in patients with poor performance status is somewhat anomalous since in many chemotherapy trials greater differences between drug effects are seen in patients with better performance status compared to those with poor performance status. The failure to see a difference in patients with good performance status may be a reflection of the small number of patients in this category. The greater treatment effect in patients with minimal prior radiotherapy may reflect less advanced disease in these patients and may also be related to the study design since patients with minimal prior radiotherapy received larger drug doses than patients with extensive prior radiotherapy. The improvement in median survival in patients with measurable disease may reflect the greater accuracy of making a proper treatment decision in a patient with measurable disease compared to a patient with nonmeasurable disease. Thus, if a patient with measurable disease shows objective response to the initial therapy, he will be continued on this therapy until evidence of disease progression; in the patient with nonmeasurable disease, judgments about the effectiveness of the initial therapy would be difficult to make and decisions about continuation of therapy may not always be made optimally.

As acid phosphatase data become available, we plan further analysis of the data to evaluate the possible role of acid phosphatase in monitoring response to therapy. Since both of the drugs evaluated in this study have demonstrated objective responses, we plan to study the effect of drug combinations in patients with advanced prostatic cancer.

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ADDENDUM

Since submission of this manuscript, this study has been closed and all patients are now being treated with adriamycin. The code for treatments shown in the text is treatment 1, 5-fluorouracil and treatment 2, adriamycin. At the present time (April 12, 1977) the objective tumor response in patients with measurable disease (complete plus partial) for initial treatment is one in 19 for 5-fluorouracil and five in 19 for adriamycin and for crossover one in eight for 5-fluorouracil and two in seven for adriamycin. Combining initial and crossover results gives two responses in 27 trials with 5-fluorouracil and seven responses in 26 trials with adriamycin; this difference is statistically significant ($P < 0.05$).

Interstitial Radiation Therapy of Cancer of the Prostate Using Iridium 192 Wires

B. Court* and D. Chassagne [1]

It is now well established that adenocarcinoma of the prostate is radiosensitive. Although high-energy external beam irradiation has been most frequently used, it requires irradiating a large volume with a high dose, which at times has been followed by complications (1,2). Interstitial radiation therapy makes it possible to give equally high doses to a small target volume. For this reason at the Institut Gustave-Roussy we initiated an after-loading technique using iridium 192 wires in plastic tubes for interstitial irradiation of patients staged as T_1 and T_2 according to the TNM system of classification of malignant tumors (UICC).

Early reports by Flocks and associates (3–7)[2] in the United States and Darget (8) in France proved that interstitial irradiation is a worthwhile modality. However, the radioactive material was difficult to handle. Pierquin and Chassagne (9), writing about cancer of the prostate, stated that "endocurietherapy techniques in continuous lines could also be foreseen, by means of Gold 198 or Iridium 192 needles or Iridium 192 wires mounted in plastic tubes. These techniques remain to be developed." Whitmore et al (10–13) developed a similar approach with a very sophisticated technique by means of permanent iodine 125 encapsulated sources. Our treatment philosophy is very similar to their indications.

Our current policy calls for visual placement of vector plastic tubes in a well-defined prostatic lesion. Moreover, it allows for the determination of nodal involvement by means of an ilio-obturator lymphadenectomy which is done at the same time.

MATERIALS AND METHODS

The patient is placed in a modified lithotomy position with the thighs flexed approximately 30°–45° on the trunk and abducted approximately 30°–45° from the central axis. In this way a double abdominal and perineal operating field is prepared. We begin by a medial subumbilical transperitoneal approach. Depending upon the type of prostatic lesion, unilateral or bilateral, ilio-obturator node dissection is carried out and a frozen histologic section of resected lymph node is prepared. Additionally, a lumboiliac node dissection with placement of metal clips in the resected node area is accomplished. Two different situations can occur. In the first case, where the patient has a nodule present within a small prostate, one proceeds with a posterior prostatorectal blunt dissection as is done in total cystectomies. This is a simple procedure if you stay well within the middle plane. It allows one to palpate the prostatic nodule between the thumb and index finger; there is no need to open the bladder wall. In the second case where the patient has both prostatic adenoma and an associated cancerous nodule, one first proceeds with a transvesical resection of the adenoma followed by placement of the vector tubes on the residual nodule present in the adenomatous bed. The radiotherapist inserts the plastic tubes by means of stainless steel needles, 17 gauge (15 cm long), from the perineum towards the prostate. The needle is pushed by the left hand and guided by the right hand, which also serves to palpate the prostatic nodule between the thumb and index finger. A loop is formed by means of an intermediate nylon obturator (Pierquin's technique for base of the tongue [14]). It is essential that the radiotherapist implants the vector tubes himself, as he is responsible for the distribution of the radioactive material and supervision. The target volume includes the whole prostate. Two or three loops with a separation factor of 10–20 mm are implanted. These wires can be placed either in a frontal or in a sagittal plane. During the operation, non-active lead wires are placed in the plastic tubes for radiologic control in order to do dosimetric calculations.

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[2]Kerr HD, Flocks RH, et al. Treatment of moderately advanced carcinoma of the prostate with radio-active gold. Presented at the Annual Meeting of the American Roentgen Ray Society, Houston, Tex, Sept 26, 1952.

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INDICATIONS

This technique applies only to stages T_1 and T_2 of the international UICC classification: T_1 = intracapsular tumor within a prostatic gland which is normal to palpation, and T_2 = tumor limited to the prostate; the nodule is smooth, bulging, but there is no invasion of the lateral rectal sulci or the seminal vesicles. N_0M_0 indicates that the patient must have previously had a normal bone scan, normal lymphangiogram, and normal bone marrow biopsy in order to eliminate all metastases. The absence of node metastases is confirmed by the ilio-obturator lymphadenectomy with frozen sections.

DISCUSSION

We would like to underline the value of interstitial radiation therapy of T_1, T_2, and N_0M_0 prostatic carcinoma. This technique is an after-loading technique with continuous iridium 192 wires. The technique allows the delivery of a dose of 6000–7000 rads to a small volume at a low dose rate; continuous irradiation is given for about 6 days.

During the placement of the vector plastic tubes, there is no irradiation of the medical staff or of the nursing personnel. This leaves enough time for an accurate localization of the target volume. Two or 3 days later the active iridium wires are loaded after obtaining a precise dosimetry. The lead wires are then replaced by the 0.3-mm diameter iridium 192 active wires.

We want to emphasize that this method can give high radiation doses to an accurately and surgically defined volume and low doses to healthy tissues.

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ANNOUNCEMENT

Medical Oncology Review Course

Pasadena, California, September 12–16, 1977

The American College of Physicians, in conjunction with the Huntington Memorial Hospital of Pasadena, California, announces the third biennial Medical Oncology Review Course scheduled to take place September 12–16, 1977, under the direction of Thomas C. Hall, MD, FACP.

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Further information may be obtained by writing to: **Mrs. Arlene Ellis, Coordinator, Medical Staff Affairs, Huntington Memorial Hospital, 100 Congress St, Pasadena, Calif 91105.**

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